# BIOLOGY AND MANAGEMENT OF *NOSEMA* DISEASE IN *MUSCIDIFURAX RAPTOR* (HYMENOPTERA: PTEROMALIDAE), A PUPAL PARASITOID OF MUSCOID FLIES

By

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Dedicated to my parents, Pamela and Teddy and in loving memory of my grandmother, Beatrice Musgrove-Pratt, and my brother, Alex.

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BIOLOGY AND MANAGEMENT OF *NOSEMA* DISEASE IN *MUSCIDIFURAX RAPTOR* (HYMENOPTERA: PTEROMALIDAE), A PUPAL PARASITOID OF MUSCOID FLIES

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*Muscidifurax raptor* is a pupal parasitoid of house flies and other filth flies. They are important natural enemies in integrated fly management programs against muscoid flies associated with poultry and livestock production. This parasitoid has been found to be infected with microsporidia in laboratory cultures, in commercial rearing facilities and also in the field where it causes a chronic disease, that reduces fitness and hampers its use as a biological control agent. Studies designed to manage this disease in insect cultures showed that heat shock treatment of infected parasitoid eggs within host puparia at 50°C for 45 minutes resulted in 100% cure and a relative survival of 18%. Heat shock treatment of infected eggs within puparia at 40°C for 1, 3, 5 and 7 hours did not result in significant reduction in the infection level. Continuous rearing of freshly parasitized house fly pupae with infected and uninfected *M. raptor* at temperatures of 15, 20, 25, 30

and 32°C showed that infected *M. raptor* had a significantly longer development time than uninfected ones. The differences in development time provided windows for collecting uninfected *M. raptor*. Also, continuous rearing of freshly parasitized house fly pupae with infected *M. raptor* at elevated temperatures of 30°C and 32°C did not cause any significant reduction in infection levels although it resulted in significant reduction in the spore concentration of the emerging adults. A 3% solution of albendazole, rifampicin and a mixture of the two when fed to infected adult *M. raptor* resulted in 20, 40, and 20% reduction respectively, in the infection level of the emerging progeny of host puparia that were exposed to the parasitoids on day 7 post treatment. Host range tests on non-target organisms showed that the spores of *N. muscidifuracis* readily germinate in a wide range of hosts without causing infection. However they caused infection in *Mucidifurax raptorellus, Spalangia gemina, S. cameroni, S. endius, S. nigroaenea* and *S. cameroni*. The infection in all the *Spalangia* spp. were light and the parasite was transmitted vertically only in *M. raptorellus* and *S. gemina*.

# CHAPTER 1
# REVIEW OF LITERATURE AND RESEARCH OBJECTIVES

## General Introduction of Microsporidia

The Microsporidia are a group of highly specialized eukaryotic spore-forming obligate intracellular protozoan parasite (Weiss, 2000). They belong to the phylum Microsporidia, which is defined as obligate intracellular protists parasites without mitochondria that form unicellular spores, each with a polar filament (Sprague and Vavra, 1977). The phylum consists of approximately 144 genera and over 1000 species (Wittner and Weiss, 1999). They are known to infect all five classes of vertebrate and almost all invertebrate phyla, including groups from the kingdom Protoctista, such as Ciliophora, Myxozoa, and Apicomplexa (Canning, 1990). Balbiani first recognized them as a distinct group in 1882, but the first microsporidia to be described was *Nosema bombycis* Nägeli, 1857, after it was recognized as the etiological agent of pebrine disease of silkworm (Sprague and Vavra, 1977; Canning and Lom, 1986; Canning, 1990; Becnel and Andreadis, 1999). This disease caused a great decline in the silkworm industry in Europe in the mid-nineteenth century (Canning, 1990).

Microsporidia are the smallest eukaryotes known, and possess the smallest eukaryote genome identified so far, and in many cases they are smaller than bacterial genome (Bidere *et al.*, 1995; Vivares *et al.*, 1996). The genome size of several microsporidia has been determined and varies from 2.9 Mb to 19.5 Mb (Biderre *et al.*,

1994; Vivares *et al.*, 1996). The small genome size of microsporidia may be an evolutionary strategy for tightly packaging its genetic information into its genome or it could be that they have lost genetic information as a result of their parasitic lifestyle, since they depend on their host cells for many metabolic pathways (Weiss and Vossbrinck, 1999). Microsporidia lack many features that are considered to be universal for eukaryotes. They lack mitochondria, centrioles, a classical Golgi apparatus, and peroxisomes (Weiss and Vossbrinck, 1999; Keeling and McFadden, 1998). They also lack an 80S ribosomal RNA, which is a characteristic of eukaryotes, but possess a 70S rRNA that is typical of prokaryotes. They are the only eukaryotes that have been reported to lack an internal transcribed spacer 2 (ITS2) in their rRNA gene sequences. In addition, the 5.8S rRNA sequence is fused with the beginning of the large subunit 23S rRNA, a characteristics that is common in bacteria (Vossbrinck and Woese, 1986; Weiss and Vossbrinck, 1998). Because the fused 5.8S-23S rRNA is unique to microsporidia and prokaryotes, this feature has been cited to support the notion that microsporidia are primitive eukaryotes (Keeling, 1998). However this could also be interpreted as an adaptation for their obligatory parasitic lifestyle.

A diagnostic character of all microsporidia is the possession of an extrusion apparatus, consisting of a polar tube or polar filament that is attached to the inside of the anterior end of the spore by an anchoring disc (Weiss, 2001). The polar filament is the apparatus used in the infection or invasive process. It forms coils around the sporoplasm ranging from 4 coils to as many as 44 in *Nosema apis* (Boucias and Pendland, 1998). In the presence of appropriate stimulus in the gut or other host tissues, the polar filament is explosively everted to form a hollow tube that penetrates the host membrane (Frixione *et*

*al.*, 1992; Frixione *et al.*, 1994; Weiss, 2001). This brings the sporoplasm into contact with the host cell cytoplasms. Conditions that elicit spore germination vary among species, and may include pH shifts, dehydration followed by rehydration, various cations and anions, temperature, ultraviolet radiation. Inhibitors of spore germination may include magnesium chloride, ammonium chloride and low salt concentrations (Undeen, 1990; Undeen and Epsky, 1990; Frixione *et al.*, 1997; Weiss, 2001).

Taxonomy and Phylogeny

The microsporidia have undergone extensive taxonomic revision over the years, since Balbiani first recognized them as a distinct group in 1882. It has been elevated to the status of phylum (the Microsporidia or Microspora, depending on the author) by Weiser (1977, 1985), Sprague (1982), Sprague and Vavra (1977) and Issi (1986). Although there are several classification systems, the one developed by Sprague is the most widely used and accepted (Brooks, 1988; Canning, 1990).

The taxonomy of microsporidian genera and species is based on their natural host, ultrastructural spore characteristics such as size, shape, and the number of nuclei per spore (monokaryon or diplokaryon), the number of polar filament coils, interface with the host cell during development and mode of cell and nuclear division (Canning and Lom, 1986; Didier *et al.*, 1998; Didier *et al.* 2000, Weiss and Vossbrinck, 1999). Sprague and Becnel (1999) have provided a checklist of 144 available generic names of the microsporidia. Morphological characters alone may be inadequate for the taxonomy of microsporidia, and other tools such as molecular techniques are used to further classify the microsporidia. The first molecular phylogenetic analysis on the gene sequences of the rRNA of a microsporidian was with *Vairimorpha necatrix* (Vossbrinck *et al.*, 1987). In

their studies, Vossbrinck *et al.* (1987) and Kamaishi *et al.* (1996) indicated that microsporidia are ancient in origin and may have diverged from the eukaryotic branch before the occurrence of mitochondrial symbiosis.

There is mounting evidence to support the notion that the microsporidia are closely related to the fungi and should be placed with them (Keeling, 1998; Weiss, 2001). Studies have shown that microsporidia possess genes for the heat shock 70 kDa proteins (hsp 70), a chaperone protein usually associated with the mitochondrial compartment. This suggests that at one point in their evolutionary history they did possess mitochondria, which have been lost secondarily. Also the alpha and beta-tubulin gene sequences found in microsporidia are more closely related to those of fungi than to other protozoans (Erlind *et al.,* 1996; Keeling *et al.,* 2000). Furthermore, the enzymes thymidylate reductase and dihydrofolic acid reductase are enzymes encoded by two distinct genes in microsporidia, fungi and animals, whereas they are usually a single protein in plants and protists (Vivares *et al.,* 1996). Microsporidia also show similarities to the fungi in mitosis and meiosis and possess chitin and trehalose like the fungi (Keeling *et al.,* 1998). They are also sensitive to some antifungal drugs. Mitochondrion-derived genes, such as HSP70 genes appears to suggest contradictory phylogenies, probably due to the fact that sequences of its genes tend to be highly divergent (Keeling and McFadden, 1998). This rapid evolution rate makes it difficult to accurately determine their phylogenetic position.

Importance of Microsporidia

The majority of microsporidia infect insect hosts and are the most important protozoan pathogens of insects (Brooks, 1988). The threat the microsporidium *Nosema bombycis* posed to the silkworm industry has already been mentioned. They also cause *Nosema* disease in the bee industry where they can cause great economic loss from honey yield if not properly managed (Fries *et al.*, 1984; Fries, 1993). Infected bees have a shorter life span than do healthy bees and the queens have reduced fecundity due to degenerate ovaries and atrophied oocytes (Liu, 1992; Wang and Moeller, 1970). Chronic infections caused by microsporidia may also play an important role in the natural regulation of insect host population (Boucias and Pendland, 1998; Canning, 1990; Kluge and Caldwell, 1992).

Microsporidia are also among the most common undetected pathogens in many insect cultures and can pose serious problems in the rearing of many insects and mites. When present they often cause chronic or debilitating disease, which results in reduced fecundity and longevity of their host (Becnel and Geden, 1994; Geden *et al.*, 1995).

The fact that microsporidia can cause disease in animals makes them potential biopesticides. *Nosema locustae* was the first microbial agent to be developed as a biopesticide for locust and grasshopper control (Henry, 1981; Johnson, 1997; Lomer *et al.*, 2001). It has been registered in the U.S. by the Environmental Protection Agency as a microbial pesticide (Brooks, 1988). *Nosema pyrausta* has also been examined as a potential microbial pesticide against the European corn borer (Lewis and Lynch, 1978; Windels *et al.*, 1976). European strains of *Vavraia* and *Nosema* isolates have been introduced into the U.S. as classical biological control agents against the gypsy moth,

*Lymantria dispar* (Jeffords *et al.*, 1989). The microsporidium, *Edhazardia aedes* has potential as a biopesticide against *Aedes aegypti,* a major vector of dengue (Becnel and Johnson, 2000). In an enclosed study, inoculative and inundative releases of *E. aedes* resulted in effective dispersal of the parasite and caused high levels of mortality that eventually eliminated a population of *A. aegypti* (Becnel and Johnson, 2000).

Microsporidia have long been recognized as important pathogens in fish (Shaw and Kent, 1999). Outbreaks have led to massive mortality and important economic losses in tropical fresh water fish, commercial fish farming, and the sport fishing industry (Shaw and Kent, 1999). The increase in aquaculture worldwide has led to the description of many new species of fish microsporidia and has also led to increased risks of epizootics (Canning, 1990; Shaw and Kent, 1999). The first known infection of a mammal by a microsporidium was *Encephalitozoon cuniculi*, infecting rabbits (Wright and Craighead, 1922). Since then, microsporidia have been found in a wide range of mammalian hosts including, rats, mice, household pets, such as dogs, various carnivores, and primates including man (Canning and Lom, 1986; Wasson and Peper, 2000). Microsporidia were first reported as an etiologic agent in humans by Matsubayashi *et al.* (1959). Since then reports about human infection were rare until the AIDS pandemic of the early eighties, during which it was frequently reported. Microsporidiosis is now a common infection in HIV patients and also in other immunocompromised patients such as organ transplant patients, with prevalence rates ranging from 2-70% (Weber *et al.*, 1994). The most common clinical presentation of microsporidiosis is gastrointestinal infection resulting in diarrhea and malabsorbtion (Costa and Weiss, 2000). Other infection sites can include the reproductive, respiratory, muscle, excretory, and nervous tissue (Weber *et al.*, 1994;

Wittner and Weiss, 1999). About 14 known species infect humans, the most common being *Enterocytozoon bieneusi, Encephalitozoon intestinalis*, and *Encephalitozoon hellem*.

Importance of pteromalids in biological control of muscoid flies

The Pteromalidae are a large assemblage of minute black or metallic green or bronze insects that parasitize a wide variety of hosts (Borror *et al.*, 1992). They are in the superfamily Chalcidoidea. The family is not defined by any unique character or combination of characters, but membership is mainly determined by elimination from other well-defined families (Gibson, 1993). Most of the species are placed in two large and poorly defined subfamilies, the Miscogastrinae and the Pteromalinae. They have a worldwide distribution, with about 845 nominal genera and 4115 nominal species (Gibson, 1993). In North America there are about 340 species (Borror *et al.*, 1992).

The family contains many of the most important parasitoids of synanthropic muscoid filth flies associated with the poultry, dairy and cattle industries (Axtell and Arends, 1990; Geden *et al.,* 1995; Morgan, 1981). Filth flies commonly associated with poultry, beef, and dairy cattle production cause significant economic loss in production facilities worldwide (Rutz and Patterson, 1990). Stable flies feed on cattle blood and reduce weight gain and also lower feed conversion efficiency (Catangui *et al.*, 1993; Floate *et al.*, 2000), whereas house flies can spread disease and are a significant nuisance and public health problem. Parasitoids that attack fly pupae are important natural enemies of filth flies and offer a promising and environmentally friendly method of control. Many of them can be easily and inexpensively mass-produced for inundative or inoculative releases to augment existing natural control (Morgan, 1981). Several commercial

insectaries are now mass-producing pteromalids, especially *Muscidifurax* spp. for biological control programs. Millions of these species are sold by commercial insectaries for releases throughout North America (Legner, 1994). The pteromalids have been successful in suppressing house fly and other filth fly populations in areas where they have been released (Geden *et al.*, 1992b; Legner and Dietrick, 1974; Morgan *et al.*, 1975).

Host - Parasitoid - Microsporidia Interactions.

Parasitoids developing in hosts that are infected with microsporidia or other pathogens such as viruses, bacteria and fungi may be directly affected at the organismic level by their development or at the population level through influence of the pathogen on the population of their hosts (Brook, 1993). The effects of parasitoids developing in infected hosts can be inconsequential, detrimental or beneficial to the host population depending on the effects of the parasitoids and pathogen on each other (Tamashiro, 1968). Detrimental effects of microsporidia on parasitoids may result in altered host tissue that are physiologically unsuitable for parasitoids development. The microsporidia may also directly invade and multiply within tissues of the parasitoids (Brooks, 1993). Hyperaparisoids may also be susceptible to the microsporidia from its host. For example the hyperparasitoid *Haplaspis nanus* and *H. aerator* are susceptible to *Nosema mesnili* infecting their host *Cotesia glomerata* (Blunck, 1954, 1952). Parasitoids may be susceptible to microsporidian infection from their host and may be able to transmit it transovarially to their progeny (Tables 1-1). There are other instances in which parasitoids are infected with microsporidia to which their hosts are not susceptible to the infection (Table 1-2), and others in which the relationships has not been determined

(Table 1-3). Parasitoid larvae developing in host infected with microsporidia may have reduced larval survival, reduced pupation or failure to emerge to adult. Emerged adults may be smaller in size (Laigo and Tamashiro, 1967; Laigo and Paschke, 1968; Cossentine and Lewis, 1986) or with distorted sex ratio (Laigo and Paschke, 1968). The development time may also be shortened, as was observed in the tachinid *Bonnetia comta* in the larva of *Agrotis ipsilon* infected with *Vairimorpha necatrix* and *Vairimorpha* species (Cossentine and Lewis, 1986).

Benel and Andreadis (1999) have listed 69 genera of microsporidia that infect 12 orders of insects of which 42 are diptera. The effect of microsporidia on diptera are similar to that observed in many parasitoid systems. The effect of the microsporidian results in reduced fecundity and survivorship. The microsporidian *Octosporea muscaedomesticae* infects a large number of muscoid flies and causes serious pathological effects on them. In the Australian sheep blowfly *Lucilia cuprina* infected with *O. muscaedomesticae*, the posterior epithelial cells are damaged (Krammer, 1966). The number of offspring emerging were also significantly reduced and infected females did not live as long as uninfected ones (Smallridge *et al*., 1995). However, males did not show significant difference in their survival and were equally capable of fertilizing eggs as uninfected males (Smallridge *et al*., 1995; Smallridge *et al*., 1990).

Biology of *Muscidifuracis raptor*

*Muscidifurax raptor* (Fig.1-1), Girault and Sanders, 1910, is a pupal parasitoid of muscoid flies that breed in animal waste and garbage. It belongs to the family Pteromalidae, which includes many parasitoids that attack dipterous pupae (Table 1-4). It is biparental, solitary, and ectophagous (Kogan and Legner, 1970) and also exhibits

arrhenotokous parthenogenesis. It is one of the most abundant house fly pupal parasitoids found in manure in poultry and dairy farms in North Carolina (Rueda and Axtell, 1985). It is nearly cosmopolitan in distribution, although it has not been reported from Asia. In North America, it is found in New York, North Carolina, South Carolina, Florida, Illinois, Missouri, Nebraska, California, and Wisconsin. In Canada, it has been reported in Alberta and New Brunswick. It is also found in Central America, the West Indies, Middle East, Europe, Australia, and in the Pacific areas including Hawaii.

The general color of adult *M. raptor* is black and the abdomen is dark brown. The female body length is about 1.9-2.5 mm with a mean of about 2.1 mm. The male is 1.7 – 2.2 mm with a mean of about 1.8 mm. (Rueda and Axtell, 1985).

The female lays her eggs by drilling a hole through the host puparium with her ovipositor and depositing a single egg on the external surface of the host. The eclosed larva kills the host and feeds externally on the host pupa within the puparium. There are three instars, which last 10-13 days followed by the pupal stage that lasts for about 5-7 days at 26°C. The life cycle from egg to adult is completed in about 17 to 22 days, (Legner and Gerling, 1967). The males emerges about 12 to 48 hours earlier than females. Geden (1997) determined the development times at various temperatures and found that the females took an average of 19.5 days to complete development at 25°C, whereas the males took an average of 17.2 days at the same temperature.

Effect and importance of *Nosema muscidifuracis* on *M. raptor*

Work by Becnel and Geden (1994), Geden *et al.* (1995), and Zchori-Fein *et al.* (1992) shed some light on the host-pathogen relationship of *Nosema muscidifuracis*. The parasite is found in all life stages of *M. raptor* and causes a chronic disease in the adult. The pathogen infects the midgut epithelium, Malpighian tubules, ovaries, fat body and muscle tissues of larvae and adults with massive numbers of spore in the tissues (Fig 1-2). Infected *M. raptor* females produce about 10% as many progeny as uninfected females and lived half as long as uninfected parasitoids (Geden *et al.*, 1995). Infected parasitoids also have a reduced attack rate killing fewer host pupae than uninfected parasitoids. This reduction in fitness or vigor of the parasitoid hinders its effectiveness in biological control efforts to manage fly problems in poultry and livestock establishments. Because there is no apparent physical manifestation of the disease in infected parasitoids regular screening of insects in cultures must be conducted. It is of utmost importance that commercial rearing facilities also take steps to ensure that diseased organisms are not sold. This would prevent the spread of microsporidia in the field and also ensure the effectiveness of these parasitoids when they are released in biological control programs.

Biology and life cycle of *Nosema muscidifuracis*

The transmission cycle of *Nosema muscidifuracis* involves both horizontal and vertical transmission. Both cycles of transmission (Fig 1-3) are necessary for the maintenance and persistence of the pathogen in its host and for transfer to subsequent generations. Spores are present in all life stages of the host including the eggs. The parasite is transmitted horizontally from one host to another through cannibalistic

behavior of the immatures, when an uninfected immature larva cannibalizes and feeds on an infective dose of spores from infected larvae in superparasitized pupae (Geden *et al.,* 1995; Becnel and Geden, 1994). The adult females feed by piercing the pupal host with their ovipositor and ingesting the released fluids (Becnel and Geden, 1994). The adults may feed on hosts parasitized by *M. raptor*, which may result in the adult cannibalizing an immature. If the immature is infected, the adult may feed on an infective dose of spores and would subsequently become infected for life.

Various life stages of *N. muscidifuracis* are shown in Figs.1-5-14. In the larvae and adults, two classes of diplokaryotic spores are produced, which are differentiated from each other by the number of polar filament coils present. One spore class has a short polar filament of approximately five turns (Fig 1- 7) and the other had a longer polar filament of approximately 9-10 turns (Fig 1-9). The spore with the short polar filament is probably responsible for spreading the infection within the host (autoinfection), since they appear to germinate immediately after formation (Becnel and Geden, 1994). The second spore class with the longer polar filament does not germinate immediately after formation and has a thicker endospore. It is presumably an environmental spore and is responsible for the transmission of the parasite to a new host. In the presporulation development, uninucleate, binucleate and diplokaryotic cells were present throughout the developmental sequences. Diplokaryotic meronts are predominant throughout development. Multiplication is predominantly by binary fission. Sporogony is disporoblastic and the early sporoblasts are elongated. The spore sizes are ovoid and measured $5.4\ 0 \pm 0.5 \times 3.0 \pm 0.2\ \mu m$, slightly smaller than those observed in the eggs which measured approximately $6.0 \pm 0.8 \times 3.2 \pm \mu m$ (Geden and Becnel, 1994).

In the eggs, vegetative stages as well as spores are present. The spores found in the eggs were unique and are the third class of spore found in the development cycle of *N. muscidifuracis*. This spore had long polar filaments that made about 15-16 turns (Fig.1-11) about the posterior vacuole (Becnel and Geden, 1994). The function of these spores is most likely for horizontal transmission (*per os*) to new hosts.

Research Objectives

The research objectives of this dissertation were the following:

1. To determine host specificity and host range of *Nosema muscidifuracis*: Can they infect other pteromalids? Can they infect organisms other than Hymenoptera?

2. To determine the differences in the development times of infected *M. raptor* and uninfected *M. raptor* and to exploit these differences as a strategy to separate infected *M. raptor* from uninfected *M. raptor*.

3. To determine whether heat shock therapy can be effective to cure infected *M. raptor* of microsporidia without causing unacceptably high mortality and also to determine whether rearing *M. raptor* at elevated temperatures would cure infected *M. raptor* of microsporidia without causing unacceptably high mortality.

4. To determine whether the drugs rifampicin and albedazole can cure infected *M. raptor* of microsporidia.

Fig. 1-1. *Muscidifurax raptor* (Hymenoptera: Pteromalidae) ovipositing on house fly pupa host. Photo, courtesy of Dr. J. Butler

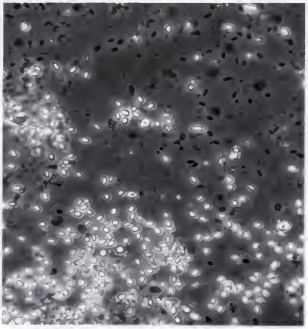


Fig. 1-2. Diplokaryotic spores of *N. muscidifuracis* from *M. raptor*. Photo, courtesy of Dr. J Becnel

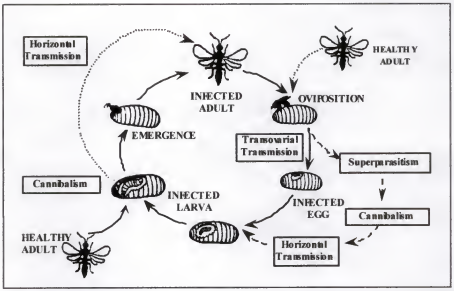



Fig. 1-3. Transmission cycle of *Nosema muscidifuracis*. The parasites are transmitted horizonatally, when infected immatures are cannibalized by uninfected immatures within superparasitized host pupa and also when uninfected adults feed on infected immatures. From Geden *et al.* 1995.

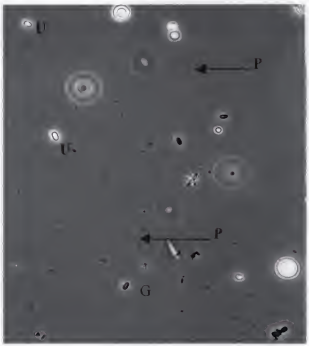



Fig. 1-4. Diplokaryotic spores of *N. muscidifuracis.* showing long polar tube (P) of germinated spore (G) and ungerminated spore, U. Photo courtesy of Dr. J. Becnel

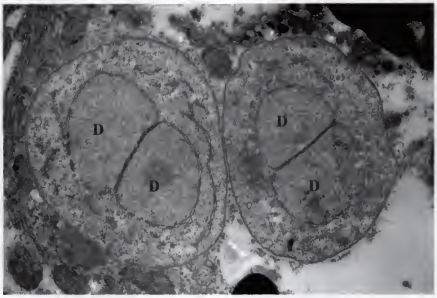



Fig. 1-5. Early diplokaryotic sporont of *N. Muscidifuracis* showing diplokaryotic nuclei, D. From Becnel and Geden 1994

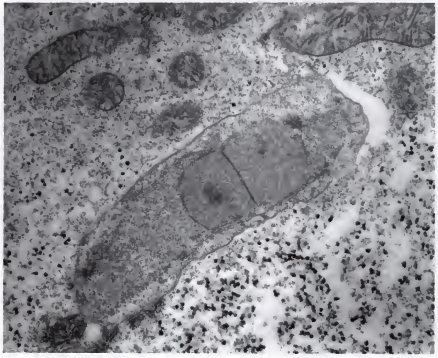


Fig. 1-6. Elongate diplokaryotic meront of *N. muscidifuracis* From Becnel and Geden 1994

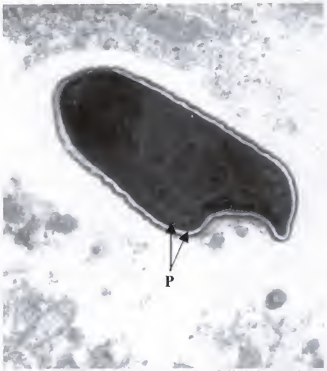



Fig. 1-7. Electron micrograph of diplokaryotic spore of *N. muscidifuracis* in *M. raptor* adult with short polar filament of about 5 coils, P. From Becnel and Geden 1994

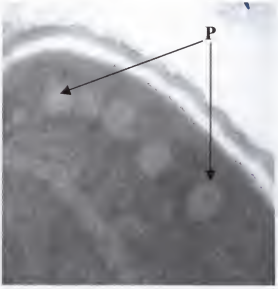



Fig. 1-8. Close –up of electron micrograph of diplokaryotic spore of *N. muscidifuracis* in *M. raptor* adult with short polar filament of about 5 coils, P. From Becnel and Geden 1994

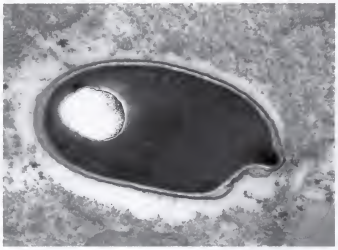


Fig 1-9. Electron micrograph of diplokaryotic spore of *N. muscidifuracis* in *M. raptor* adult with long polar filament of about 9-10 turns. From Becnel and Geden 1994

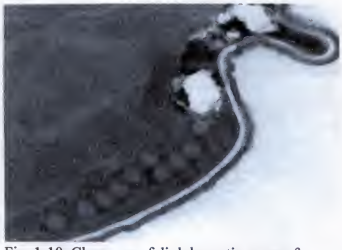


Fig. 1-10. Close –up of diplokaryotic spore of *N. muscidifuracis* in *M. raptor* adult with long polar filament of about 9-10 turns P. From Becnel and Geden 1994

Fig 1-11. Electron micrograph of diplokaryotic spore of *N. muscidifuracis* in *M. raptor* eggs with long polar filament of about 15-16 turns. From Becnel and Geden 1994

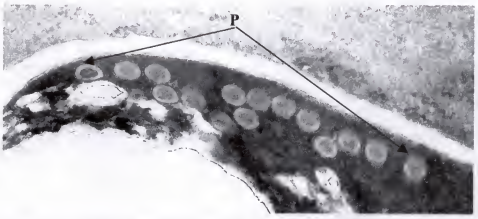



Fig. 1-12. Close up electron micrograph of dioplokaryotic spore of *N. Muscidifuracis* in *M. raptor* eggs with long polar filament of about 15-16 coils. From Becnel and Geden 1994

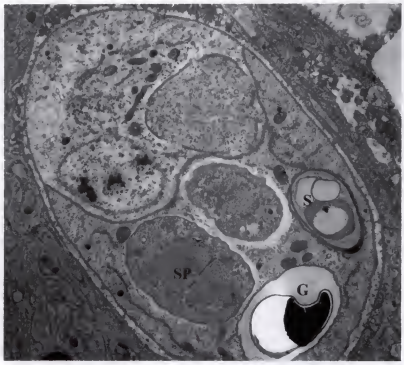



Fig. 1-13. Electron micrograph of infected cell of *M. raptor* showing sporont, SP. spore, S and germinated spore, G. From Becnel and Geden 1994

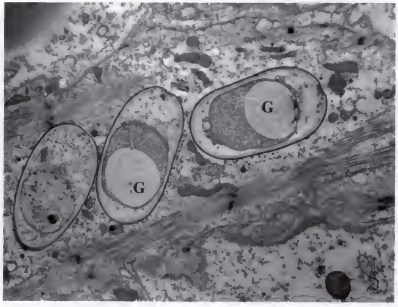



Fig. 1-14. Electron micrograph of infected cell of *M. raptor* showing germinated spore, G. From Becnel and Geden 1994

Table1-1. Parasitoids directly susceptible to the microsporidia infecting their hosts.[1]

| Parasitoid | Host | Microsporidium | References |
|---|---|---|---|
| Braconidae | | | |
| *Ascogaster quadridentata* | *Laspeyresia pomonella* | *Nosema carpocapsae* | Huger and Neuffer (1978) |
| *Bracon mellitor* | *Anthonomous grandis* | *Nosema gasti* | Bell and McGovern (1975) |
| *Chelonus annulipes* | *Ostrinia nubilalis* | *Nosema* spp. | York (1961) |
| *Cotesia glomerata* | *Pieris brassicae* | *Nosema mesnili* | Blunck (1952, 1954); Tanada, (1955); Larsson (1979) |
| | *Aporiae crataegi* | *Nosema aporivora* | Lipa (1957) |
| *Cotesia marginiventris* | *Heliothis zea* | *Vairimorpha* sp. | Hamm *et al.* (1983) |
| *Cotesia rubecula* | *Pieris brassicae* | *Nosema mesnili* | Blunck (1952, 1954) |
| *Macrocentrus grandii* | *Ostrinia nubilalis* | *Nosema pyrausta* | York (1961); Andreadis (1980, 1982); Cossentine and Lewis (1987) |
| Chalcididae | | | |
| *Dahlbomius fuscipennis* | *Neodiprion swainei* | *Cystosporogenes pristihorae* | Smirnoff (1971) |
| *Dibrachys cavus*[2] | *Cotesia glomerata* | *Nosema mesnili* | Blunck (1952, 1954) |
| *Spilochalcis side*[2] | *Campoletis sonorensis* | *Nosema campoletidis* | Brooks (1973); McNeil and Brooks (1974) |
| Eulophidae | | | |
| *Pediobius foveolatus* | *Epilachna varivestis* | *Nosema epilachnae* | Own and Brooks (1986) |
| | | *Nosema varivestis* | Own and Brooks (1986) |

Table1-1 Continued.

| Parasitoid | Host | Microsporidium | References |
|---|---|---|---|
| Inchneumonidae | | | |
| *Campoletis sonorensis* | *Heliothis zea* | *Nosema heliothidis* | Brooks and Cranford (1972) |
| *Gelis tranfuga* | *Cotesia glomerata* | *Nosema mesnili* | Blunck (1952, 1954) |
| *Haplaspis aerator* | *Cotesia glomerata* | *Nosema mesnili* | Blunck (1952, 1954) |
| *Haplaspis nanus* | *Cotesia glomerata* | *Nosema mesnili* | Blunck (1952, 1954) |
| *Hemiteles simillimus* | *Cotesia glomerata* | *Nosema mesnili* | Blunck (1952, 1954) |
| *Hyposoter ebenius* | *Pieris brassica* | *Nosema mesnili* | Hostounsky (1970) |
| *Pimpla instigator* | *Cotesia glomerata* | *Nosema mesnili* | Hostounsky (1970) |
| *Thysiotorus brevis* | *Cotesia glomerata* | *Nosema mesnili* | Blunck (1952, 1954) |
| Pteromalidae | | | |
| *Catolaccus aeneoviridis*[2] | *Campoletis sonorensis* | *Nosema campoletidis* | Brooks (1973); McNeil and Brooks (1974) |
| Trichgrammatidae | | | |
| *Trichgramma evanescens*[3] | *Ostrinia nubilalis* | *Nosema pyrausta* | Huger (1984) |
| *Trichogramma nubiliale* | *Ostrinia nubilalis* | *Nosema pyrausta* | Sajap and Lewis (1988) |

[1] From Brooks (1993)
[2] Species of hyperparasites
[3] Species of egg parasitoids

Table 1-2. Microsporidia of parasitoids in which the host of the parasitoid is not susceptible

| Microsporidium | Parasitoid | Host of parasitoid | References |
|---|---|---|---|
| *Nosema muscidifuracis* | *Muscidifurax raptor*[1] | *Musca domestica* | Becnel and Geden (1994)<br>Geden *et al.* (1995) |
| *Nosema campoletidis* | *Campoletis sonorensis*[2] | *Heliothis zea* | Brooks and Cranford (1972) |
| *Nosema cardiochilis* | *Cardiochilis nigiceps*[3] | *Heliothis virescens* | Brooks and Cranford (1972) |

[1] Pteromalidae
[2] Ichneumonidae
[3] Braconidae

Table 1-3. Undescribed microsporidia from parasitoids in which the microsporidia, parasitoid and host interrelationships have not been determined.

| Microsporidia | Parasitoid | Host of parasitoid | References |
|---|---|---|---|
| *Microsporidium* sp. | *Muscidifurax* spp<br>*Nasonia vitripennis*<br>*Spalangia cameroni*<br>*Spalangia endius*<br>*Pachycrepoideus vindemiae* | *Musca domestica* | Dry *et al.* (1999) |
| *Nosema* sp. | *Encarsia* | *Bemisia aregentifoli* | Sheetz *et al.* (1997) |

Table 1-4. Some dipteran hosts of *Muscidifurax raptor*[1].

| Family | Species |
|---|---|
| Muscidae | *Musca domestica* |
| | *Fannia canniculatis* |
| | *Fannia femoralis* |
| | *Haematobia sp.* |
| | *Musca autmnalis* |
| | *Ophyra leucstoma* |
| | *Stomoxys calcitrans* |
| Calliphoridae | *Cochliomyia macellaria* |
| | *Phormia regina* |
| Sarcophagidae | *Ravina querula* |

[1] Adapted from Rueda and Axtell (1985)

CHAPTER 2
TRANSMISSION TESTING WITH *NOSEMA MUSCIDIFURACIS* (MICROSPORIDIA: NOSEMATIDAE) IN OTHER PTEROMALID PARASITOIDS AND NON - TARGET ORGANISMS.

## Introduction

The microsporidia is a large group of obligate intracellular parasites that are known to infect all classes of vertebrate and almost all invertebrate phyla. Their most common hosts are arthropods and fish (Canning, 1990). Successful transmission of the pathogen from one host to another or from parent to offspring is necessary for the pathogen to persist in their host population (Andreadis, 1987). Transmission of microsporidia involves both horizontal and vertical components. Horizontal transmission occurs when the pathogen is transmitted from one individual to another within or between generations but not from parent to offspring (Steinhaus and Martignoni, 1970; Andreadis, 1987). Vertical transmission is the direct transfer of the pathogen from parent to offspring typically through the host egg. Transmission via the egg is termed transovum and occurs in two distinct ways, either within the eggs (transovarially) or on the surface of the eggs (contamination) (Becnel and Andreadis, 1999). Horizontal transmission represents the major transmission path utilized by the majority of microsporidia infecting invertebrates (Dunn *et. al.*, 2000). Most microsporidia incorporate both transmission routes for sustaining the infection in the host populations. However, there are some that use only the transovarial route (Bunheim and Vavra, 1968; Bunheim, 1971; Ni *et. al.*,

1997; Terry *et. al.*, 1997). *Nosema muscidifuracis* is a pathogen of *Muscidifurax raptor*, a parasitoid of muscoid flies (Becnel and Geden, 1994). The microsporidium adversely affects the fitness of *M. raptor*, reducing longevity and fecundity (Geden *et al.*, 1995). Becnel and Geden (1994) have described the morphological feature, and developmental sequences of *N. muscidifuracis* and examined its host-parasite relationship. *N. muscidifuracis* is horizontally transmitted through an oral route when a healthy immature cannibalizes an infected immature within a superpasitized host or when a healthy adult cannibalizes an infected immature within its host puparia during host feeding (Geden *et al.*, 1995). The pathogen is also transmitted transovarially within the eggs of infected mothers. Venereal or paternal transmission is rare and has not been demonstrated in *Muscidifurax raptor* (Geden *et al.*, 1995), but has been reported in *Nosema plodia,* (Kellen and Lindergren, 1971) and *Nosema kingii,* (Armstrong 1978).

Microsporidia were generally considered host-specific, and new species were frequently described because of their presence in new hosts such as *Nosema apis*, and *N. bombycis* (Brooks, 1988; Tanada and Kaya, 1993). However, bioassays have shown that some microsporidia can infect a wide range of hosts from different insect families or orders (Hall, 1952; Tanabe and Tamashiro, 1967; Brooks, 1970; Undeen and Maddox, 1973). Most of the infections of a particular species are found within the same orders, families or genera. No entomogenous microsporidia are known to infect healthy homoeothermic hosts. However, *Nosema algerae*, a mosquito microsporidium, has been induced to infect cooler body sites in the white mouse and in immunocompromised mice (Undeen and Alger, 1976; Trammer *et al.*, 1997).

The first step in the infection process after the ingestion of infective spores by susceptible insects is the germination of the spores in the insect's digestive tract (Undeen, 1976). Many factors are known to affect germination, including pH, ions, osmotic pressure, temperature, and inhibitors (Undeen, 1990; Undeen and Epsky, 1990; Frixione *et al.*, 1994). In many intracellular pathogens, the processes involved in the ingress of the pathogen into the host cell dictate the tropism or specificity of the disease agent (Boucias and Pendland, 1998). Microsporidia posses a unique infection apparatus, the polar filament, which is triggered during germination of the spore and is extruded directly into the host cell. They are therefore equipped to bypass the normal receptor-ligand interaction that is present in other intracellular pathogens (Boucias and Pendland, 1998). Therefore factors that elicit or inhibit the polar extrusion mechanism may dictate the host and tissue specificity of the microsporidia (Boucias and Pendland, 1998). Although physiological host specificity data produced in the laboratory from bioassays may not always reflect the true ecological host specificity (Solter and Maddox, 1998), they are still used to predict the ecological host range of an organism (Federici and Maddox, 1996; Solter *et al.*, 1997). The host specificity and host range of *Nosema muscidifuracis* are unknown. Preliminary investigations have shown that the house fly host was not involved in the transmission process (Geden *et al.*, 1995). The focus of this study is to determine whether *N. muscidifuracis* could be transmitted to other pteromalid parasitoids and to selected alternate dipteran and lepidopteran hosts.

## Material and Methods

### Insect Colonies

#### Host (*Musca domestica*) rearing.

House fly colonies are routinely maintained at the USDA-ARS Center for Medical, Agricultural and Veterinary Entomology (CMAVE), in Gainesville, Florida to serve as host for parasitoids and also for other research endeavors. The immatures are fed a mixture of wheat bran, corn meal, alfalfa and water. The dry diet is mixed with water in a ratio of 3: 1 by volume. A volume of 3ml of eggs is placed on 7 liters of the diet in a tray and kept in a room at 24-26°C and relative humidity of 60-70%Rh. After about 5 days the pupae are floated with water and dried in a blower. They are placed in 1 liter cups and irradiated in a cesium 127 source for 40 minutes to kill the pupae and thereby eliminating the problem of emerged flies which are not parasitized. The parasitoids are able to survive on the dead pupae. Some pupae are not irradiated and left to emerge to maintain the colony. Adult house flies are kept at 24-28°C and at 60-70%RH. They are placed in cages with water and fed a mixture of dried skimmed milk powder, egg yolk and sugar. Eggs are collected by wrapping some of the spent media in a moist black cloth and placing the cloth in the cage for oviposition.

#### Parasitoid rearing

Infected and healthy colonies of *Muscidifurax raptor* are also maintained at the USDA-ARS-CMAVE laboratories. The infected colony was originally collected from dairy farms in New York in 1987. The uninfected colony was established by eliminating the disease from this colony by heat shock treatment (Chapter 4). The parasitoids are placed in a 43 × 43 × 20 cm plexiglas cages with cotton sleeve to facilitate easy handling

of the insects. The plexiglas cage is placed in a 72 × 46 × 55 cm large wooden box which is inside a walk-in rearing chamber maintained at 23-26 °C. Parasitoids are given irradiated house fly pupae as hosts for feeding and oviposition two to three times weekly, in a paper can that is 9 cm in diameter and 4.5 cm in height which contains about 5-6 thousand pupae. After the pupae have been in the cage for 2-3 days, the paper can is capped with a cover that has a wire mesh top and the insects are shaken from it. The paper can which now contains developing parasitoids is capped, labeled and kept in the wooden cage until emergence. After emergence the paper can in placed in the plexiglas cage and the old paper can containing the host pupal cases of emerged parasitoids is discarded. The healthy colonies are kept separately from the infected colonies and are routinely screened at least once a month for infection.

Non-target testing.

Diptera: Culicidae

Three species of mosquitoes, C*ulex quinquefasciatus*, *Aedes aegypti*, and *Anopheles quadrimaculatus* were obtained from the mosquito rearing facility at CMAVE. Second instars (100 per group) of each species were placed in small plastic containers. Infected *M. raptor* (N=40) were macerated in a glass tissue grinder in 3ml of deionized water. The spores were counted with a hemacytometer and resulted in a spore concentration of $7.1\times10^6$ spores/ml. The spore solution was made to give a final spore concentration of $7\times10^4$ spores/ml in 100 ml of water for each species of mosquito. Mosquito diet was added to the water and the mosquitoes were exposed to the spores. Controls were set up for each species as above, but with no spores added into the water. After one hour, five larvae from each group were removed and the midgut dissected to

check for germination of spores. The rest were allowed to go through normal development to the adult stage. The adult mosquitoes were dissected and checked for spores. Giemsa stains of some of the adults were made and checked under a phase contrast microscope for vegetative stages of the parasite.

Diptera: Muscidae

*Musca domestica* larvae and adults

House fly larvae that were in the second instar were counted in 8 batches of 50. Four batches were placed on fly media that was mixed with *N. muscidifuracis* spores at a concentration of $3.9 \times 10^6$ spores/ml in 1 oz plastic cups. The other 4, which served as controls, were placed in fly media without any spores. After two hours, a sample of 5 larvae was removed from the group that were given spores and dissected to check for germination of spores within the midgut.

Four groups of newly emerged house fly adults (40 per group) were placed in 1-liter plastic containers. About 100 infected *M. raptor* were ground in a glass tissue grinder with 4ml of deionized water to make a spore solution of $3.9 \times 10^7$ spores/ml. The spore solution was pipetted into two portions of 2ml each and one was placed in a small plastic container containing 10 mls of water and small pieces of styrofoam, which acted as resting and/or landing sites for the flies to feed. A drop of honey weighing about 0.2g was added to the spore solution and placed in each of the 2 containers. The two containers serving as negative controls were set up as described above, except that 100 healthy *M. raptor* adults were macerated and mixed with a drop of honey and fed to the flies. After two hours a sample of 10 flies from each group was dissected and checked for evidence of germination within the midgut. The spore solution was replaced with fresh

spores on the second day. A sample of 10 flies was removed from each group on the fourth day and dissected to check for infection. At the end of day 7, the remaining flies were dissected and examined for infection.

*Hydrotaea aenescens*

Adults of the black dump fly, *Hydrotaea aenescens*, were similarly tested as described for *M. domestica.*

Lepidoptera

The corn earworm, *Helicoverpa zea*, was obtained from the Behavior and Biocontrol Research Unit of the USDA-CMAVE in Gainesville, Florida. Two spore concentrations of $3.6 \times 10^6$ and $7.8 \times 10^6$ spores/ml were made by macerating 200 *M. raptor* adults infected with *N. muscidifuracis* to give a low and high concentration, respectively. The spore solution was forced through cotton in a syringe to remove chitinous debris. Artificial diet for *H. zea* in 1 oz plastic cups (90 total) were also obtained from the Behavior and Biocontrol group at USDA-CMAVE. They were divided into three groups of 30 each to serve as the controls, low and high concentration, respectively. For each group, about 40 $\mu$l of either low or high concentration spores were place on the surface of the diet. For the controls, healthy *M. raptor* were macerated as described above and 40 $\mu$l of the solution were placed on the surface of the diet. One second instar larva was placed in each of the 1 oz plastic cups with the treated diet. After 5 days, 5 larvae were randomly selected from each group and dissected to check for infection. The remaining larvae were allowed to develop to the adult stage. The adults were closely examined for deformation. They were dissected and checked for any signs of infection.

Pteromalidae

Uninfected colonies of six species of pteromalids, namely, *Muscidifurax raptor*, *Muscidifurax raptorellus*, *Spalangia gemina*, *Spalangia endius, Spalangia nigroaenea*, and *Spalangia cameroni* were obtained from colonies maintained at USDA- CMAVE in Gainesville, Florida. Uninfected *M. raptor* (400 females and 200 males) that were 1-2 days old were held for 12-18 hours without hosts in a 1-liter plastic container with a cotton sleeve for easy access and handling. Two groups were made, one to serve as a positive control and the other as negative control. Infected *M. raptor* (N=100) were macerated in a glass tissue grinder in 4 ml of deionized water as described above. A spore solution of $7.6\times10^6$ spores/ml was made. Fifty $\mu$l of the spore solution were pipetted onto a clean glass microscope slide and a drop of honey was placed in it. It was stirred with a glass rod and covered with a Kimwipe® tissue paper and placed in the container with the parasitoids. Two strips of Kimwipe® paper measuring 8 cm in length and 2 cm in width were also smeared with the spore solution and placed inside the container. The negative controls were set as described above, but instead of using infected *M. raptor*, uninfected *M. raptor* were used. The same procedure was repeated for all the species. In the case of the *Spalangia* spp., 250 females and 100 males were used in each test. There were no males available for *Spalangia endius*. Mortality data were collected every other day. At least 25 females and 15 males were collected from the container and was dissected and checked for infection on days 3, 5, and 7 for the *Muscidifurax* species and an additional sample on the day 9 was done for the *Spalangia* species. All treatments were given house fly pupae every other day at a host: parasitoid ratio of 10:1 for feeding and oviposition.

The pupae for day 7 of *Muscidifurax* species and day 9 of the *Spalangia* species were kept until emergence to check for transovarial transmission. The infection levels of the emerged progeny was recorded. The experiment was replicated at least two more times with other batches of spores and cohorts of parasitoids.

The results for the were analyzed by G tests of independence comparing the mortality and infection of the various parasitoids that were fed with honey and spores with those that were fed with honey alone.

## Results

None of the alternate hosts or non-target organism tested for transmission of *N. muscidifuracis* became infected (Table 2-1). Results of dissection of the larvae of the mosquito species showed dark opaque spores, indicative of germinated or immature spores. The gut contents of the mosquito species, therefore, seemed to be conducive for germination of the spores of *N. muscidifuracis*. There were no apparent physical changes or signs of deformities between the treated and the controls for all stages. Dissection of pupae and adults did not show any spores, and giemsa stains of the adults revealed no vegetative stages of *N. muscidifuracis*.

*Musca domestica* also did not show signs of infection in any stage of development. There was evidence of germination in the gut of the larvae and adults. The spores did not seem to have any effect on the mortality of houseflies in all stages. Similar results were observed for the black dump fly *Hydrotaea aenescens*, with germination of spores in the gut of adults. Infection with corn earworm was also negative in all life stages. There were no deformities in the adult and *N. muscidifuracis* appeared to have no effect on the mortality of the larvae.

Mortality data of pteromalids

Mortality of *M. raptor* females fed with *N. muscidifuracis* spores was higher than those fed without spores on all days (Table 2-2). This increase in mortality ranged from 15 - 45% by day 7 post-exposure. By day 7 the mortality for the female parasitoids that fed on *N. muscidifuracis* spores was 44.2% while mortality was 24.1% among parasitoid that did not feed on spores. For days 1 and 3 for *M. raptor* there were no significant differences in the mortality between spore fed females and controls. However for days 5 and 7 the differences in the mortality were significant (Table 2-2). Differences in mortality between the spore-fed and control males were not as high as those of the females. In fact there were not significant differences between the spore-fed males and the control for all days. Controls had higher mortality than spore-fed males up to day 5 post-exposure (Table 2-2), but by day 7 the mortality for spore-fed group was 48.8% and that for the controls was 42.5%. However these differences were not significant (Table 2-2).

Mortality for *M. raptorellus* was higher for spore-fed parasitoids than controls on all days and for both sexes (Table 2-3). Male mortality on days 5 and 7 was more than twice that of the females for both spore-fed and control group. Similar mortalities were observed for the males on days 1 and 3 for both the spore-fed and control groups and they were not significantly different (Table2-3). However, by day 5 the mortality for the spore-fed was about twice that of the controls. Mortality of the control for the males at day 7 was 70.4%, while that for the control males were 92%. The differences in the mortality for days 5 and 7 were significant for both males and females.

In general, feeding on spores had little effect on mortality of the *Spalangia* species, which were longer-lived than either of the *Muscidifurax* species (Tables 2-4-2-7). Mortality of spore-fed *S. gemina* were higher than the controls on day 9 for both sexes and these differences were significant (Table 2-4). There was no significant difference between the spore-fed *S. nigroaenea* and the controls for both sexes at all days (Table 2-5). Similarly there was no significant difference between the spore fed *S. cameroni* and the controls for both sexes at all days except for the males on day 9 in which the difference in the mortality was significant (Table 2-6). There were significant mortality differences for *S. endius* for days 5, 7, and 9 (Table 2-7).

Infection level

Both *Muscidifurax* species developed detectable infections 3 days post-exposure to *N. muscidifuracis* spores. From the insects sampled on day 3, *M. raptor* and *M. raptorellus* showed infection levels of 8.9 and 12.3%, respectively (Fig. 2-1). Only *Spalangia nigroaenea* showed any infection (1.3%) by day 3 for the *Spalangia* species tested (Fig. 2-1). By day 5 *M. raptor* had the highest percent infection of 30.7% among all species (Fig. 2-2). *Spalangia gemina* had an infection of 25.7% whereas that of *M. raptorellus* was 15.3%. All the other *Spalangia* species had infection levels of less than 10%, with *S. endius* showing only a 2% infection level (Fig. 2-2). By day 7 the infection level of all the species increased. The infection for *M. raptor* was 48.8% whereas that for *M. raptorellus* was 34.5%. Infection levels for the *Spalangias* spp. were 37.3, 35.5, 16.2, and 11.5% for *S. gemina*, *S. nigroaenea*, *S. cameroni* and *S. endius*, respectively (Fig. 2-3). By day 9, *S. gemina* and *S. nirgoaenea* showed similar infection levels of 38.2 and

37.1%, respectively. The infection levels of *S. cameroni* and *S. endius* were 19.6 and 12.9 respectively.

Females of *M. raptor* had a much higher infection level (59.4%) compared to the males (36.5%), while with *M. raptorellus* males and females has similar infection levels of about 36% for day 7 (Table 2-8). With the exception of *S. gemina* all the *Spalangia* males had a higher infection level than their female counterpart for day 9 (Table 2-8).

Transovarial transmission.

Both species of *Muscidifurax* transmitted *N. muscidifuracis* to their progeny, with large amounts of spores visible in the alimentary canal, malpighian tubules and fat body of the emerged adults. The spore morphology in the progeny was similar to that in the parents. *M. raptor* showed a vertical infection rate of 43.3% while that of *M. raptorellus* was 27% (Table 2-9). Among the *Spalangia* species, only *S. gemina* transmitted the parasite vertically, with an infection level of 8.5% of the progeny (Table 2-9). *S. endius, S. cameroni* and *S. nigroaenea* all showed no evidence of vertical transmission, even though 13-32% of the parentals were infected.

## Discussion

The spores of *Nosema muscidifuracis* germinate in the midguts of all the mosquito species tested. The pH of the midgut of several mosquito species has been found to be highly basic, ranging from 8.6 to 9.4 (Christophers, 1960). Undeen (1976) also found the midgut contents to be basic in several mosquito species. *In vitro* studies of *N. muscidifuracis* have indicated a predisposition to germinate in a wide range of pH media ranging from 7-10.7 (unpublished data). In deionized water solution this species

also exhibits a predisposition to germinate spontaneously without any apparent stimuli. This characteristic prevented the use of purified spores in the bioassays, because initial efforts with purified spores indicated that many spores germinated in the purification process. Although the spores germinated in the gut of all the mosquitoes species and the other dipteran insects tested, germination did not lead to infection in the hosts. This demonstrates that lack of infection was not due to unfavorable gut conditions for germination. However, one cannot say for certain whether the germination of spores in the gut was due to stimuli from the gut, since it was not possible to distinguish spores that germinated spontaneously from one that may have germinated due to some stimuli from midgut conditions. The extrusion of the polar filament to the target cell, which is one of the first steps in the infection process, appears to be undirected. There is no evidence to suggest that the extruded polar filament can selectively detect and penetrate its host (Boucias and Pendland, 1998). Many different microsporidia have been triggered to germinate in the midguts of insects. The mosquitoes were exposed to *N. muscidifuracis* at a spore concentration of $6.0\times10^4$ spores/ml; this is comparable to the $3.2\times10^4$ spores/$cm^2$ of *Nosema algerae* that Undeen (1976) used to infect 4 species of mosquitoes.

There were no apparent mortality effects of *N. muscidifuracis* in any of the mosquito species tested, in the immatures and adults of *Musca domestica*, or in *Hydrotaea* and *Helicoverpa* species. There were no differences in the developmental period nor were there any morphological abnormalities noticed. This implies that *Musca domestica* hosts are not involved in transmission cycle of *Nosema muscidifuracis* and supports the observation made by Geden *et al.* (1995). This host-parasitoid-microsporidian interrelationship is similar to the results observed by Brooks and

Cranford (1972) in which they described two new microsporidia, *Nosema campoletidis* and *N. cardiochilis*. *N. campoletidis* infects the ichneumonid *Campoletis sonorensis*, but did not infect *Heliothis zea* in cross infectivity tests (Brooks and Cranford, 1972). Similarly, *N. cardiochilis*, a microsporidian parasite of *Cardiochiles nigiceps* was not infective to either *H. zea* or *H. virescens* (Brooks and Cranford, 1972). However, there was no effect on the development time of the parasitoid, *Campoletidis sonorensis* nor were there any effect on the fecundity and longevity of the parasitoid in contrast to what was observed in *Nosema muscidifuracis* in which the parasite affected the parasitoids survival and fecundity (Geden *et al*., 1995). In a similar study McNeil and Brooks (1974) found that the hyperparasitoids *Catolaccus aeneoviridis* and *Spilochalcis side*, though they were susceptible to the microsporidian *Nosema campoletidis*, it was not detrimental to them in terms of adult longevity. Also while *N. campoletidis* was transmitted tranovarially in *C. aeneoviridis* it was not in *S. side* because microsporidian development was halted (McNeil and Brooks, 1974).

Mortality effects of *N. muscidifuracis* were more apparent with *Muscidifurax raptor* and *M. raptorellus*, especially on days 5 and 7 in both species, which was significantly different for both sexes on both days except for the *M. raptor* males (Table 2-2). There was higher percentage mortality of about 40% in the females of both species when they were fed with *N. muscidifuracis* spores compared to only about 24% of the females when they were fed without spores. There was 45, and 42% more mortality for the females of *M. raptor* and *M. raptorellus,* respectively when they were fed on spores. Zchori-Fein *et al.* (1992) showed that the infection of *M. raptor* with *N. muscidifuracis* had a detrimental effect. Infected *M. raptor* parasitoids lived half as long,

produced up to half as many progeny as uninfected parasitoids and had a longer developmental time (Zchor-Fein *et al.*, 1992). The mortality data for the males of the *Muscidifurax* species did not differ greatly between those that fed on *Nosema* spores and those that did not. The disease, therefore, seems to have more of an effect on the females than on the males. The disease in the males seems to serve no purpose in its natural transmission and appears to be a dead end since there is no paternal or venereal transmission (Geden *et al.*, 1995). However, in the laboratory, crowding often leads to superparasitism, and the infection in the male may help in the amplification of the disease. This is because healthy adult females may acquire the infection by feeding on infected immatures. Also a healthy immature female may cannibalize an infected immature male and acquire the infection for life and be able to transmit it to its progeny transovarially (Becnel and Geden,1994; Geden *et al.*, 1995). This scenario is presumably rare in nature where superparasitism levels are typically low. Another possible method in which the infection could be spread by males and females is through their feces, which have been found to contain spores. It is possible that they may excrete on pupae or near pupae and contaminate them with spores which may be picked up by other healthy parasitoids when they attempt to feed on such pupae.

The high mortality in the males of *M. raptorellus*, 92% for those that were fed spores and 70.4% for the control at day 7, is probably due to the inherently short lifespan of this species compared to the *Spalangia* species. However, the effect of *N. muscidifuracis* may account for the 23% difference in the mortality. The mortality effects of the *N. muscidifuracis* in the *Spalangia* were not as obvious as in the *Muscidifurax* species. Mortality was similar for both the spore-fed and controls for most species.

All the pteromalids tested were susceptible to infection with *N. muscidifuracis*. Infection, as determined by the appearance of spores, was first visible in the *Muscidifurax* species by day 3 post-exposure to spores. Of the *Spalangia* species, only *S. gemina* showed any infection by day 3. The infection level in *Muscidifurax* was 8.9 and 12.3%, respectively, for *M. raptor* and *M. raptorellus* on day 3. The rapid manifestation of the disease might suggest that *M. raptorellus* is also a natural host for *Nosema muscidifuracis*. By day 7, about half of the *M. raptor* were infected whereas only about a third of the *M. raptorellus* were infected. *S. gemina* and *S. nigroaenea* showed infection levels of 37.3 and 35.5%, respectively (Fig. 2-3). *S. cameroni* and *S. endius* showed 16.2 and 11.5% , respectively (Fig. 2-3). Although spore loads were not quantified in the tests, examination of infected parasitoids indicated that spore loads were profoundly greater in the *Muscidifurax* species than in any of the *Spalangia* species. Indeed, infection in *Spalangia* were typically very light and required careful examination to detect. This could also be a reflection that the *Spalangia* species are not natural hosts of *Nosema muscidifuracis*. There were also visible differences in the spore sizes between the *Muscidifurax* and the *Spalangia* species tested. While the spore sizes for the *Muscidifurax* were similar in size to the spores that were used to infect them, the spore size of the microsporidia in all the *Spalangia* species were much smaller in size. This parallels the observation made by Brooks and Cranford (1972), in which the spore sizes of *Nosema heliothidis* was significantly different when it was infecting *Heliothis zea* than when it infected the parasitoid *Campoletis sonorensis*.

Vertical transmission was observed in both *Muscidifurax* species, but of the *Spalangia* species, it was only observed in *S. gemina*. In surveys that were undertaken to

find the prevalence of microsporidia in cultures of pteromalid parasitoids of muscoid flies, almost all the colonies of *Muscidifurax* were found to be infected with microsporidia, but no infections were found in any of the *Spalangia* colonies (Zchori-Fein, 1992). It was thought that the *Spalangia* species might not be susceptible. Since then, Dry *et. al.* (1999) have found *S. cameroni* and *S. endius* with microsporidia from the field. We have recently also found *S. gemina* and *S. cameroni* to be infected with what appears to be a distinctive microsporidian with patterns of transmission similar to that of *N. muscidifuracis* (Unpublished data).

The reason why there was no vertical transmission in the *Spalangia* spp. is not known, but one could speculate that the low spore production in the *Spalangia* species could be a factor. It is also likely that the infection did not spread to the ovaries. There were many more spores in the progeny of *M. raptor* than in the parents. This could be due to the fact that *M. raptor* acquired the infection from the egg stage and had a much longer developmental period for amplification (18 days) than the infection in the adult stage (7 days). In general the spore load seems to be very low in the egg stage and increases with each successive developmental stage of larvae, pupae, and adult (Zchori-Fein *et al.*, 1992). At the adult stage there seems to be an exponential increase in spore production. It's easy to envision this as an evolutionary strategy of the microsporidia to perpetuate itself, because if there are too many spores in the egg stage it would deplete nutritional reserves and kill the eggs and eventually kill the microsporidia itself. Canning *et al.* (1982) have observed that vertical transmission can be detrimental if the microsporidia kills the egg, and light infection of the eggs are necessary for the successful transmission of the pathogen to subsequent generations. In previous studies,

vertical transmission of *N. muscidifuracis* in *M. raptor* was observed to be 100% efficient (Geden *et al.*, 1995; Zchori-Fein *et al.*, 1992), but those experiments were conducted with females from infected colonies that had acquired the infection prior to adult emergence. The vertical transmission efficiency of 43% (Table 2-9) obtained in the present study may have been due to the method of infection or to the fact that not all the parent females used in the test were infected with *M. raptor*.

In summary, these results indicate that *N. muscidifuracis* from *M. raptor* can easily be transmitted to *M. raptorellus*, resulting in a disease that closely resembles that seen in the natural host. Transmission to *Spalangia* species resulted in light infections with either low (*S. gemina*) or zero rates of vertical transmission presumably due to lack of infection in the ovaries or very low spore load in them. These results suggest that *Nosema* diseases found in the field populations of *Spalangia* species represent distinct diseases caused by other species of parasites. The study also shows the potential of the microsporidium *N. muscidifuracis* to spread from one host species to another new species, as was seen in *M. raptorellus* and *S. gemina*, which were able to transmit the parasite transovarially to their progeny. This could be problematic especially for intensive mass cultures involving multiple parasitoid species.

Table 2-1. Infection status of alternate host after exposure to spores of *Nosema muscidifuracis*.

| Species | Spore concentration Spores/ml | Infection status |
|---|---|---|
| *Aedes aegypti*[1] | $6.0\times10^4$ | No infection |
| *Anopheles quadrimaculatus*[1] | $6.0\times10^4$ | No infection |
| *Culex quinquefasciatus*[1] | $6.0\times10^4$ | No infection |
| *Musca domestica*[1] | $7.8\times10^6$ | No infection |
| *Hydrotaea aenesecens*[3] | $7.8\times10^6$ | No infection |
| *Helicoverpa zea*[2] | $3.6\times10^6$ | No infection |
| *Helicoverpa zea*[2] | $7.8\times10^6$ | No infection |

[1] All three life stages were found to be negative;
[2] The larval and adult stages were negative.
[3]Adults only.

Table 2-2. Mortality of *M. raptor* (SE) that were fed (for 7 days ) with either a honey solution containing *N. muscidifuracis* spores or honey solution without spores.

| Day | Sex | Mean (SE) % mortality of parasitoids fed on: | | $\chi^2$ |
|---|---|---|---|---|
| | | Honey + spores[1] | Honey only[2] | |
| 1 | Female | 1.2 (0.9) | 0.9 (1.2) | 0.6ns |
| 3 | | 9.3 (4.9) | 7.9 (8.4) | 2.3ns |
| 5 | | 25.6 (10.7) | 16.6 (14.1) | 50.2** |
| 7 | | 44.2 (3.5) | 24.1 (9.4) | 152.7** |
| 1 | Males | 0.8 (1.0) | 1.5 (1.7) | 1.0ns |
| 3 | | 11.3 (5.2) | 12.0 (7.4) | 0.1ns |
| 5 | | 26.3 (8.1) | 30.5 (18.6) | 1.8ns |
| 7 | | 48.8 (10.6) | 42.5 (16.4) | 3.2ns |

[1.] Adult parasitoids allowed to feed *ad lib* on a suspension of honey and water with $7.6\times10^6$ spores/ml from macerated *M. raptor*.

[2.] Adult parasitoids fed on honey, water and macerated uninfected *M. raptor*.

ns, $P > 0.05$; *, $P < 0.05$; ** $P < 0.01$ (G-tests of independence comparing mortality of parasitoid that were fed with honey and spore with those that were fed with only honey)

Table 2-3. Mortality of *M. raptorellus* (SE) that were fed (for 7 days) with either a honey solution containing *N. muscidifuracis* spores or honey solution without spores.

| Day | Sex | Mean (SE) % mortality of parasitoids fed on: | | $\chi^2$ |
|---|---|---|---|---|
| | | Honey + spores[1] | Honey only[2] | |
| 1 | Female | 4.1 (0.1) | 2.3 (1.3) | 4.6* |
| 3 | | 10.4 (2.6) | 9.3 (3.8) | 0.6ns |
| 5 | | 23.4 (0.6) | 15.5 (5.3) | 15.9** |
| 7 | | 40.5 (2.5) | 23.3 (8.5) | 55.3** |
| 1 | Males | 2.8 (0.8) | 2.5 (1.5) | 0.0ns |
| 3 | | 11.8 (1.8) | 9.6 (0.4) | 1.8ns |
| 5 | | 52.8 (1.2) | 27.5 (12.5) | 27.2** |
| 7 | | 92.0 (8.0) | 70.4 (26.4) | 34.6** |

[1] Adult parasitoids allowed to feed *ad lib* on a suspension of honey and water with $7.6\times10^6$ spores/ml from macerated *M. raptor*.

[2] Adult parasitoids fed on honey, water and macerated uninfected *M. raptor*.

ns, $P > 0.05$; *, $P < 0.05$; ** $P < 0.01$ (G-tests of independence comparing mortality of parasitoid that were fed with honey and spore with those that were fed with only honey)

Table 2-4. Mortality of *Spalangia gemina* (SE) that were fed (for 9 days) with either a honey solution containing *N. muscidifuracis* spores or honey solution without spores.

| Day | Sex | Mean (SE) % mortality of parasitoids fed on: | | $\chi^2$ |
|---|---|---|---|---|
| | | Honey + spores[1] | Honey only[2] | |
| 1 | Female | 5.0 (4.6) | 6.2 (5.4) | 0.7ns |
| 3 | | 6.6 (5.0) | 8.8 (7.2) | 1.7ns |
| 5 | | 8.4 (6.0) | 10.4 (8.4) | 1.1ns |
| 7 | | 11.2 (8.0) | 11.4 (9.0) | 0.0ns |
| 9 | | 21.6 (16.8) | 13.6 (10.4) | 11.1** |
| 1 | Males | 7.0 (4.0) | 4.5 (3.5) | 1.2ns |
| 3 | | 9.5 (2.5) | 9.0 (8.0) | 0.0ns |
| 5 | | 14.5(0.5) | 12.0 (10.0) | 0.5ns |
| 7 | | 25.5 (10.5) | 15.5 (10.5) | 0.2ns |
| 9 | | 45.0 (27.0) | 27.0 (21.0) | 14.2** |

[1] Adult parasitoids allowed to feed *ad lib* on a suspension of honey and water with $7.6\times10^6$ spores/ml from macerated *M. raptor*.

[2] Adult parasitoids fed on honey, water and macerated uninfected *M. raptor*.

ns, $P > 0.05$; *, $P < 0.05$; ** $P < 0.01$ (G-tests of independence comparing mortality of parasitoid that were fed with honey and spore with those that were fed with only honey)

Table 2-5. Mortality of *Spalangia nigroaenea* (SE) that were fed for (9 days) with either a honey solution containing *N. muscidifuracis* spores or honey solution without spores.

| Day | Sex | Mean (SE) % mortality of parasitoids fed on: | | $\chi^2$ |
|---|---|---|---|---|
| | | Honey + spores[1] | Honey only[2] | |
| 1 | Female | 6.5 (2.8) | 6.5 (0.5) | 0.0ns |
| 3 | | 23.7 (12.0) | 25.0 (11.3) | 0.3ns |
| 5 | | 29.2 (13.5) | 29.2 (13.2) | 0.0ns |
| 7 | | 37.5 (13.8) | 34.5 (15.8) | 1.2ns |
| 9 | | 45.0 (16.0) | 39.7 (17.3) | 3.5ns |
| 1 | Males | 8.5 (3.5) | 8.5 (0.5) | 0.0ns |
| 3 | | 36.5 (16.5) | 34.5 (0.5) | 0.2ns |
| 5 | | 45.0 (18.0) | 48.5 (0.5) | 0.5ns |
| 7 | | 53.5 (16.5) | 55.5 (0.5) | 0.2ns |
| 9 | | 63.5 (17.5) | 71.5 (4.5) | 1.7ns |

[1] Adult parasitoids allowed to feed *ad lib* on a suspension of honey and water with $7.6\times10^6$ spores/ml from macerated *M. raptor*.

[2] Adult parasitoids fed on honey, water and macerated uninfected *M. raptor*.

ns, $P > 0.05$; *, $P < 0.05$; ** $P < 0.01$ (G-tests of independence comparing mortality of parasitoid that were fed with honey and spore with those that were fed with only honey)

Table 2-6. Mortality of *Spalangia cameroni* (SE) that were fed (9 days) with either a honey solution containing *N. muscidifuracis* spores or honey solution without spores.

| Day | Sex | Mean (SE) % mortality of parasitoids fed on: | | $\chi^2$ |
|---|---|---|---|---|
| | | Honey + spores[1] | Honey only[2] | |
| 1 | Female | 0.8 (0.5) | 1.3 (0.3) | 0.7ns |
| 3 | | 5.8 (1.5) | 5.7 (0.3) | 0.0ns |
| 5 | | 7.7 (2.7) | 6.8 (0.2) | 0.3ns |
| 7 | | 11.3 (3.7) | 10.0 (0.0) | 0.6ns |
| 9 | | 14.2 (3.8) | 12.5 (0.2) | 0.7ns |
| 1 | Males | 4.5 (3.5) | 5.0 (2.0) | 0.0ns |
| 3 | | 13.0 (6.0) | 10.0 (3.0) | 0.9ns |
| 5 | | 20.0 (7.0) | 19.0 (1.0) | 0.0ns |
| 7 | | 28.5 (9.5) | 23.0 (2.0) | 1.6ns |
| 9 | | 39.5 (12.5) | 29.5 (2.5) | 4.4* |

[1] Adult parasitoids allowed to feed *ad lib* on a suspension of honey and water with $7.6\times10^6$ spores/ml from macerated *M. raptor*.

[2] Adult parasitoids fed on honey, water and macerated uninfected *M. raptor*.

ns, $P > 0.05$; *, $P < 0.05$; ** $P < 0.01$ (G-tests of independence comparing mortality of parasitoid that were fed with honey and spore with those that were fed with only honey)

Table 2-7. Mortality of female *Spalangia endius* (SE) that were fed (9 days) with either a honey solution containing *N. muscidifuracis* spores or honey solution without spores.

| Day | Mean (SE) % mortality of parasitoids fed on: | | $\chi^2$ |
|---|---|---|---|
| | Honey + spores[1] | Honey only[2] | |
| 1 | 7.5 (0.5) | 5.0 (0.3) | 3.2ns |
| 3 | 16.5 (2.5) | 17.8 (2.2) | 0.4ns |
| 5 | 36.7 (10.7) | 30.0 (0.7) | 6.0* |
| 7 | 48.3 (9.3) | 37.8 (0.8) | 13.5** |
| 9 | 57.0 (9.3) | 45.8 (0.2) | 15.0** |

[1] Adult parasitoids allowed to feed *ad lib* on a suspension of honey and water with $7.6\times10^6$ spores/ml from macerated *M. raptor*.

[2] Adult parasitoids fed on honey, water and macerated uninfected *M. raptor*.

ns, $P > 0.05$; *, $P < 0.05$; ** $P < 0.01$ (G-tests of independence comparing mortality of parasitoid that were fed with honey and spore with those that were fed with only honey)

Table 2-8. Infection level of pteromalids at either 7 (*Muscidifurax* spp) or 9 (*Spalangia* spp) days post-exposure to *Nosema muscidifuracis* spores at a concentration of. $7.6\times10^6$ spores/ml.

| Species | Sex | N | Percent Infection (SE) | $\chi^2$ |
|---|---|---|---|---|
| *Muscidifurax raptor* | Female | 131 | 59.4 (9.6) | 54.7** |
| *M. raptorellus* | | 76 | 36.5 (3.8) | 161.4** |
| *Spalangia gemina* | | 63 | 42.5 (3.2) | 29.3** |
| *S. nigroaenea* | | 50 | 32.5 (3.5) | 19.5** |
| *S. cameroni* | | 60 | 16.7 (0.0) | 9.8** |
| *S. endius* | | 47 | 12.9 (1.4) | 7.0** |
| *M. raptor* | Male | 76 | 36.5 (3.8) | 26.4** |
| *M. raptorellus* | | 45 | 36.7 (3.4) | 23.1** |
| *S. gemina* | | 32 | 34.0 (7.3) | 18.6** |
| *S. nigroaenea* | | 38 | 42.0 (2.0) | 25.0** |
| *S. cameroni* | | 44 | 22.5 (2.5) | 12.8** |

$P< 0.01$ (G-tests of independence comparing infection level of parasitoid that were fed with spores with the controls)

Table 2-9. Infection level of progeny of pteromalids species given hosts on either day 7 or day 9 post-exposure to spores of *N. muscidifuracis*.

| Species | N | Percent Infection |
|---|---|---|
| *M. raptor* | 120 | 43.3 |
| *M. raptorellus* | 100 | 27.0 |
| *S. gemina* | 70 | 8.6 |
| *S. nigroaenea* | 40 | 0.0 |
| *S. cameroni* | 40 | 0.0 |
| *S. endius* | 40 | 0.0 |

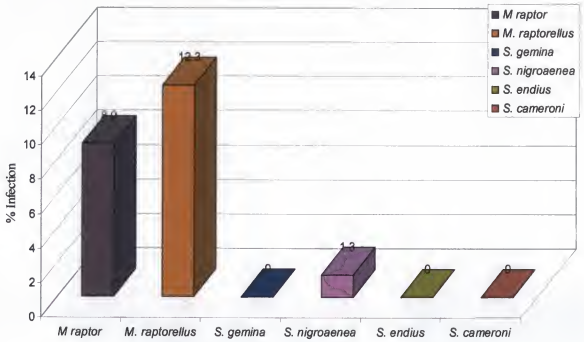



Fig. 2-1. Infection levels of various species of pteromalids at 3 days post-exposure to *N. muscidifuracis* spores.

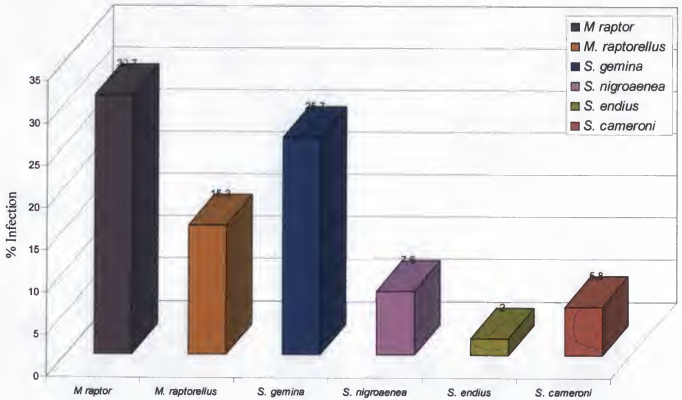



Fig. 2-2. Infection levels of various species of pteromalids at 5 days post-exposure to *N. muscidifuracis* spores.

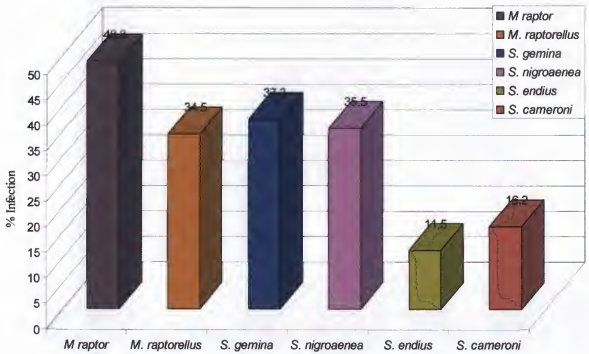



Fig. 2-3. Infection levels of various species of pteromalids at 7 days post-exposure to *N. muscidifuracis* spores.

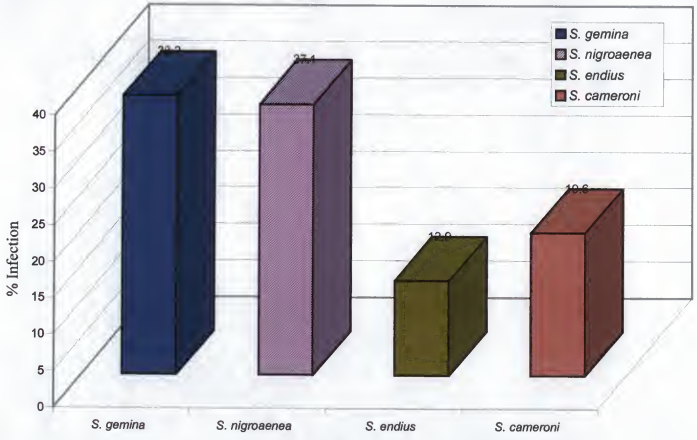



Fig. 2-4. Infection levels of various species of pteromalids at 9 days post-exposure to *N. muscidifuracis* spores.

# CHAPTER 3
# THE EFFECT OF *NOSEMA MUSCIDIFURACIS* (NOSEMATIDAE: MICROSPORIDIA) ON DEVELOPMENT TIMES OF *MUSCIDIFURAX RAPTOR* (HYMENOPTERA: PTEROMALIDAE) AT VARIOUS TEMPERATURES

## Introduction

The family Pteromalidae contains several important parasitoids for the control of muscoid flies associated with dairy and poultry production (Axtell and Arends 1990, Geden *et al.*, 1995). They can be mass-produced easily and inexpensively for inundative or inoculative releases to augment existing natural control (Morgan 1981). Sustained releases of parasitoids in this family in integrated management programs have provided control of house flies and other muscoid flies (Morgan *et al.*, 1975; Morgan 1981; Rutz and Axtell 1981; Geden *et al.*, 1992b). Several commercial insectaries are engaged in the production of pteromalids, especially *Muscidifurax* spp., to sell to the poultry and large animal production industry. However, some control programs have met with mixed results, possibly due to infection of the parasitoids with microsporidia (Geden *et al.*, 1992; Rutz and Patterson 1990; Greene 1990).

Microsporidia are a large and ubiquitous group of obligate intracellular parasites that produce spores containing a polar filament. They are found in all major animal phyla, and are among the smallest eukaryotes known. Becnel and Geden (1994) have described the host pathogen relationship for the parasitoid *M. raptor* Girault & Sanders and the pathogen *Nosema muscidifuracis*. The pathogen infects the midgut epithelium,

Malpighian tubules, ovaries and fat body of larvae and adults (Becnel and Geden 1994). The infection causes a chronic disease in adult parasitoids, resulting in reduced fecundity and longevity (Geden *et al.*, 1995). The transmission cycle involves horizontal and vertical transmission. The disease is transmitted horizontally when healthy adult parasitoids feed on infected immatures within the host puparium, and also when healthy parasitoid immatures cannibalize infected immatures within superparasitized hosts. Maternal transmission is nearly 100% efficient and there is no paternal or venereal transmission (Geden *et al.*, 1995).

The detection of microsporidia in virtually every colony of *Muscidifurax* spp. in the world, including those maintained by commercial insectaries, has implications for biological control programs because infected parasitoids have reduced longevity, searching ability, and fecundity compared to uninfected parasitoids (Zchori-Fein *et al.* 1992, Dry *et al.*, 1999). Prevalence of *N. muscidifuracis* is between 1 and 11% in *M. raptor* populations on dairy farms where no parasitoids were released, in contrast to 84% in farms where infected parasitoids were inadvertently released (Geden et. al. 1995). It is important for rigorous quality control measures to be implemented to ensure that infected parasitoids are not released in the field (Geden *et. al.,* 1995). Field surveys of pteromalids indicate that microsporidian infections of fly parasitoids are widely distributed and infections have been found in New York, Florida, Arkansas (Geden *et al.*, 1995, Dry *et al.,* 1999), South Korea and Brazil (CJG, unpublished data).

Strategies that have been used to eliminate or reduce microsporidian infection in colonies include the Pasteur method, in which family lines are obtained from individual mated females that are examined for infection after oviposition. Heat and drug therapies

also have proven to be effective in certain cases (Cantwell and Shimanuki 1969; Hsiao and Hsiao 1973; Brooks *et al.*, 1978; Briese and Milner 1986; Geden *et al.*, 1995; Sheetz *et al.,* 1997). Because infection with microsporidian pathogens is known to result in delayed development times in some insects (Habtewold *et al.*, 1995; Bauer and Nordin, 1988; Geden *et al.*, 1992b), there is also the possibility that differential development times can be exploited to isolate uninfected individuals. The objective of this study was to determine whether infection of *M. raptor* with *N. muscidifuracis* would prolong the parasitoid's development time to such an extent that developmental windows could allow the collection of uninfected parasitoids.

## Materials and Methods

*Muscidifurax raptor* females (N=400) infected with *Nosema muscidifuracis* were obtained from a colony maintained at the USDA-ARS Center for Medical, Agricultural and Veterinary Entomology (CMAVE), in Gainesville, FL. The parasitoids were provided with 2400 1- 2-day-old house fly (*Musca domestica* L.) pupae for oviposition for 24 hours at 25ºC. Similarly, 400 uninfected *M. raptor* females were collected from an uninfected colony of *M. raptor* and exposed to 2400 *Musca domestica* pupae at 25ºC for 24 hours. After exposure to the parasitoids, each set of pupae was divided into 6 groups of 400 pupae. The pupae were placed in small round paper cups (9 cm in diameter and 5 cm in depth) with a cover. Sets of pupae (i.e. 400 pupae from the infected group and 400 pupae from the uninfected group) were placed in plastic containers ($36 \times 25 \times 12$cm) containing a saturated NaCl solution to maintain constant humidity of 75 –80%RH (Winston and Bates, 1960). A group of pupae was placed inside an environmental

chamber at 15, 20, 25, 30, 32 or 34°C. Temperature and humidity conditions in the chambers were monitored with StowAway® data loggers (Onset Computer Corporation, Pocasset, MA)

The containers were checked daily for fly emergence. Flies that emerged were discarded and uneclosed pupae were counted, placed individually in standard no. 2 gelatin capsules, and returned to the chambers for parasitoid emergence. The containers were monitored twice daily at 12-hour intervals for parasitoid emergence. Emerged parasitoids were counted and sexed until emergence ceased (7 days after emergence of last parasitoid), and development time was determined for each individual. Parasitoids were crushed on a microscopic slide in a drop of water and examined at 400× for the presence of spores. The entire experiment was replicated twice with different cohorts (parasitoids from different parents) of parasitoids.

Development time data were analyzed separately for males and females by using the General Linear Models procedure of the Statistical Analysis System (SAS Institute, 1992), with temperature, infection status and temperature × infection status as the grouping variables. An *a posterori* means separation analysis was performed to examine the effect of infection on development time for each temperature. Emergence success of infected versus uninfected parasitoids was compared by conducting separate G-tests of independence for males and females at each temperature (Sokal and Rohlf, 1981). The effect of temperature on emergence success was evaluated by comparing parasitoid emergence at 25°C with emergence at other temperatures by using separate G-tests of independence for both sexes and infection status groups (Sokal and Rohlf, 1981).

## Results

No uninfected parasitoids emerged at 34°C, and only one emerged at this temperature in the infected group (data not shown). At the remaining temperatures, the average number of hosts attacked by uninfected parasitoids was 380.0 ± 5.6 pupae out of the 400 that were provided; these pupae produced 287.0 ± 21 progeny (n=10 sets of exposed host pupae). In contrast, infected parasitoids attacked 238.2 ± 37 pupae out of 400 provided and produced 127.5 ± 37 progeny (Table 3-1). Infected parasitoids produced 75.3% fewer females and 49.9% fewer males than uninfected parasitoids. The effect of infection on emergence success also was modulated by temperature (table 3-1). Infected and uninfected parasitoids had significantly lower emergence success at 15°C compared with 25°C. In contrast, exposure to 32°C resulted in significantly lower emergence of infected parasitoids of both sexes but not of uninfected parasitoids.

Development times of infected parasitoids were significantly longer than those of uninfected parasitoids for both males and females (Tables 3-2 and 3-3). This effect was significant for females at all temperatures except 15°C, and resulted in extending the development time of infected parasitoids by 6.3, 4.7, 8.3, and 11% at 20, 25, 30, and 32°C, respectively. Infected males required 6.3, 6.1, 4.8, 7.5 and 7.4% more time to develop than uninfected females at 15, 20, 25, 30, and 32°C, respectively.

The distribution of development times for females is shown in Fig. 3-1. The emergence at each temperature is calculated as a proportion of the total in order to facilitate comparison of emergence patterns of infected versus uninfected parasitoids (more healthy parasitoids emerged than infected ones). Emergence at 15°C occurred over

25 days for both infected and uninfected parasitoids, and there were no clear time intervals when only infected or uninfected individuals emerged (Fig. 3-1A). At 20°C emergence occurred over 10 days, however there was a clear separation in emergence patterns between uninfected and infected individuals. The first uninfected parasitoids emerged 12 hours before the first infected ones, and 61% of the uninfected parasitoids had emerged by day 30 compared with 10% of the infected individuals (Fig.3-1B).

At 25°C, 22% of the uninfected parasitoids had emerged in the 36 hours before emergence of the first infected individual, and 37% of the uninfected females had emerged by day 19 compared with 8% of the infected females (Fig.3-1C). At 30°C, 16% of the uninfected parasitoids had emerged in the 24 hours before emergence of the first infected individual, and 40% of the uninfected parasitoids had emerged by day 14 compared with 4% of the infected females (Fig. 3-1D). Emergence at 32°C was compressed into a short time interval, with most parasitoids emerging over a six-day period between days 12 and 17 (Fig.3-1E). Almost 60% of the uninfected parasitoids had emerged by day 14 compared with 12% of the infected parasitoids, but the time of emergence of the first individuals of both infection classes was similar.

Distribution of development times for males is shown in Fig. 3-2. In general, the patterns for males were similar to the females, with the uninfected males emerging first at all temperatures. At 15 and 20°C (Figs 3-2A and 3-2B, respectively), the first two days of emergence yielded only uninfected parasitoids. At 25, 30 and 32 °C, most of the uninfected males emerged within the first two days.

## Discussion

*Nosema muscidifuracis* causes a chronic and debilitating disease in *M. raptor* which reduces the fitness of the parasitoid with respect to reproductive potential, longevity, and ability to locate and parasitize hosts (Zchori-Fein *et al.*, 1992, Geden *et al.*, 1995). Our data on relative attack rates and progeny production by infected and uninfected *M. raptor* confirm previous observations that infected individuals have lower fecundity than healthy parasitoids, and that poor colony performance is not merely due to differential mortality of adults. Various developmental stages of the pathogen are found in the ovaries, fat bodies, Malpighian tubules and midgut epithelium (Becnel and Geden 1994). The infection of the ovaries and fat bodies of the adult females may be responsible for the reduced fecundity and longevity since the fat body is the storage site for nutrients in insects, and the females are dependent for the protein reserves needed for egg maturation (Gaugler and Brooks 1975; Mitchell and Cali 1994).

There are no gross differences in appearance between infected and uninfected *M. raptor*, although Geden *et al.*, (1992a) did observe that infected individuals were somewhat smaller-bodied than healthy parasitoids. In practice, infection of colonies usually goes unnoticed until there is a marked deterioration in colony production parameters (Geden *et al.*, 1992a). Microsporidian infections may be common in colonies of other beneficial arthropods as well. Such infections have recently been found in several *Spalangia* spp., the encyrtid parasitoid *Tachinaephagus zealandicus* (Ashmead) (unpublished data) and in colonies of predacious mites, where they also affect the overall fitness and effectiveness of these biological control agents (Bjornson 1999, M. Hoy, pers. comm.).

In the present study, infected parasitoids produced progeny with a higher proportion of males than did uninfected parasitoids. The reason for this is unclear, and may be due to poor mating success of infected males or to higher mortality among infected females in the immature stages. Because infected females contain more spores than males (Zchori-Fein *et al.*, 1992), infection may have resulted in differential mortality of females compared with males.

Our development times for uninfected *M. raptor* are in agreement with those obtained by Geden (1997) at 15, 20, 25, 30 and 32°C. We observed no emergence at 34°C, and Geden (1997) reported very low emergence at 35°C, indicating that these temperatures represent the upper limit of tolerance for *M. raptor*. The duration of parasitoid emergence was longer at the lower temperatures, spanning about 25 days at 15°C, about 10 days at 20 and 25°C, and about 8 days at 30 and 32°C. Approximately 90% of the parasitoids emerged within 4 days at 25, 30 and 32°C.

Infected parasitoids took longer to develop than uninfected parasitoids under nearly all of the test conditions (Table 3-2). Many authors have observed that insects infected with microsporidia have prolonged development times (Gaugler and Brooks 1975; Mitchell and Cali 1994; Becnel and Undeen 1992; MacVean and Capinera 1991, Srivastava and Bhanotar 1986; Habtewold *et al.*, 1995). MacVean and Capinera (1991) found that *Nosema locustae* and *Vairimorpha* sp. significantly retarded development of the early nymphal stages of host crickets. Similarly, Srivastava and Bhanotar (1986) showed that the incubation period of eggs and the duration of the first two nymphal instars were significantly prolonged in desert locusts that were infected with *N. locustae*.

Gaugler and Brooks (1975) found that sublethal infection of the corn earworm (*Heliothis zea*) with *N. heliothidis* retards larval development and results in deformed pupae. Similar results were obtained by Mitchell and Cali (1994) with *Vairimorpha nectatrix* infection in *H. zea*. In contrast, Brooks and Cranford (1972) found no obvious effects of *Nosema campoletis* in infected *Heliothis* spp., although they observed mortality effects with *N. heliothidis.*

The reason for delayed development in infected *M. raptor* is not known. Delayed development of the immature stages of insects infected with microsporidia may be due to depletion of nutritional reserves, consumption of less food material, and a reduced ability to assimilate food efficiently (Johnson and Pavlikova 1986; Veber and Jasic 1961; Thomson 1958). Based on observations made during other experiments with infected *M. raptor*, there does not appear to be a significant lengthening of the egg stage in infected colonies (CJG, unpublished data). Additional research involving dissections of parasitized hosts at various intervals would be required to determine which immature stages are most affected by infection.

There are substantial differences in development times of infected versus uninfected *M. raptor* and that these differences are large enough to exploit in efforts to manage the disease in those instances where colony infection is less than 100%. Large proportions of healthy females can be collected by retaining only those females that emerge in the first 24 hours of emergence at 25°C or during the first 48 hours of female emergence at 20°C (Fig 3-1A and Fig 3-1B). Some emerging uninfected females may mate with infected males, but the absence of paternal or venereal transmission of this pathogen makes this unimportant from the standpoint of colony health (Geden *et al.*,

1995). Our research provides a simple method for commercial insectaries and researchers to obtain uninfected females as founder stock for establishing uninfected cultures of this important beneficial insect.

Table 3-1. Emergence of uninfected *M. raptor* and of parasitoids infected *with Nosema muscidifuracis* at different temperatures.

| Sex | Temp. (°C | No parasitoids emerged | | | % emergence (relative to 25°C) | | | |
|---|---|---|---|---|---|---|---|---|
| | | Uninfected | Infected | $\chi^2$ | Uninfected | $\chi^2$ | Infected | $\chi^2$ |
| Females | 15 | 188 | 32 | 109.1**[1] | 78.7 | 6.1*[2] | 43.5 | 16.4**[2] |
| | 20 | 205 | 58 | 75.5** | 85.5 | 2.6ns | 79.4 | 1.7ns |
| | 25 | 239 | 73 | 78.6** | 100.0 | - | 100.0 | - |
| | 30 | 214 | 57 | 83.7** | 89.5 | 1.4ns | 78.1 | 2.0ns |
| | 32 | 233 | 49 | 112.5** | 97.5 | 0.1ns | 67.1 | 4.7* |
| Males | 15 | 234 | 112 | 18.9** | 69.9 | 18.0** | 61.5 | 16.8** |
| | 20 | 277 | 148 | 31.6** | 82.7 | 5.5* | 81.3 | 3.5ns |
| | 25 | 335 | 182 | 34.9** | 100.0 | - | 100.0 | - |
| | 30 | 346 | 171 | 42.1** | 104.2 | 0.2ns | 97.2 | 0.1ns |
| | 32 | 311 | 141 | 51.4** | 92.8 | 0.9ns | 78.2 | 5.2* |

[1] G-test of independence (df=1) comparing emergence of infected versus uninfected parasitoids at each temperature. **, P<0.01; *, P<0.05, ns,P>0.05

[2] G-test of independence (df=1) comparing emergence of infected or uninfected parasitoids relative to emergence at 25°C. **, P<0.01; *, P<0.05, ns,P>0.05

Table 3-2. Development times (egg to adult) of male and female *M. raptor* and *M. raptor* infected with *Nosema muscidifuracis* at different temperatures.

| Sex | Temp°C | Mean (SE) dev time (days) | |
|---|---|---|---|
| | | Uninfected | Infected |
| Females | 15 | 67.05 (0.32)a[1] | 67.86 (0.85)a |
| | 20 | 30.40 (0.12)a | 32.30 (0.24)b |
| | 25 | 19.64 (0.07)a | 20.67 (0.17)b |
| | 30 | 14.61 (0.07)a | 15.82 (0.12)b |
| | 32 | 14.19 (0.07)a | 15.76 (0.16)b |
| Males | 15 | 61.35 (0.31)a | 65.21 (0.58)b |
| | 20 | 27.83 (0.10)a | 29.54 (0.14)b |
| | 25 | 17.85 (0.06)a | 18.71 (0.06)b |
| | 30 | 13.39 (0.04)a | 14.40 (0.06)b |
| | 32 | 13.16 (0.05)a | 14.14 (0.07)b |

[1] Means followed by the same letter within a row are not significantly different at P= 0.05 based on the Ryan- Einot-Gabriel- Welsch Multiple Range test.

Table 3-3. Analysis of variance for the effects of temperature and infection with *Nosema muscidifuracis* on development times of male and female *Muscidifurax raptor*.

| Sex | ANOVA effect | df | F | Significance level |
|---|---|---|---|---|
| Females | Temp. | 4, 1347 | 320.93 | <0.0001 |
| | Infection | 1, 1347 | 28037.12 | <0.0001 |
| | Temp * Inf. | 4. 1347 | 1.42 | 0.2260 |
| Males | Temp. | 4, 2252 | 35364.91 | <0.0001 |
| | Infection | 1, 2252 | 317.83 | <0.0001 |
| | Temp * Inf. | 4. 2252 | 32.78 | <0.0001 |

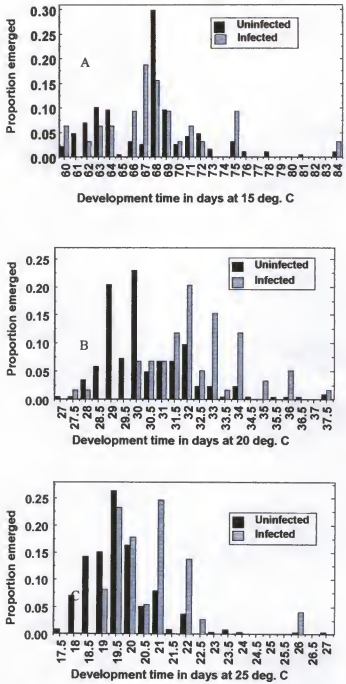
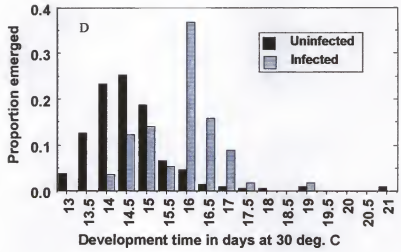



Fig 3-1. Distribution of development time of infected and uninfected females *M. raptor* reared at various temperatures. (A) development time in days at 15°C. (B) development time in days at 20°C. (C) development time in days at 25°C. (D) development time in days at 30°C. (E) development time in days at 32°C



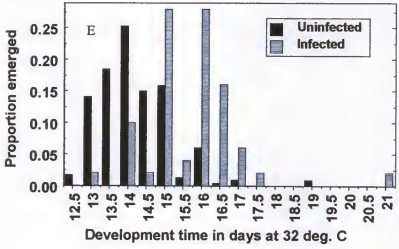




Fig 3-1. Continued

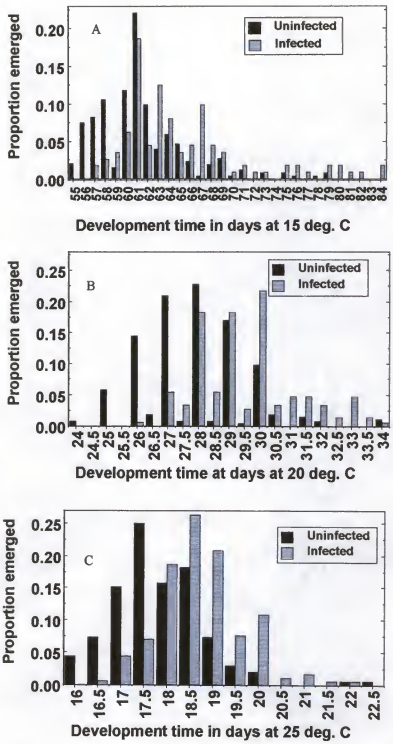
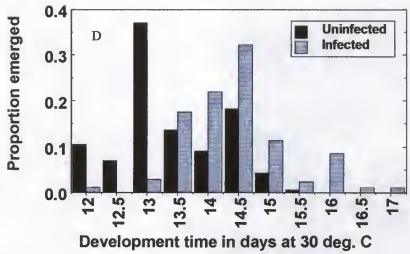



Fig 3-2. Distribution of development time of infected and uninfected males *M. raptor* reared at various temperatures. (A) development time in days at 15°C. (B) development time in days at 20°C. (C) development time in days at 25°C. (D) development time in days at 30°C. (E) development time in days at 32°C



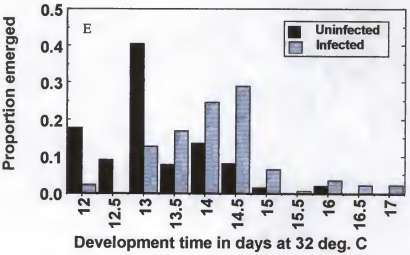




Fig 3-2. Continued

CHAPTER 4
EVALUATION OF REMEDIATION METHODS FOR *NOSEMA* DISEASE IN *MUSCIDIFURAX RAPTOR* (HYMENOPTERA: PTEROMALIDAE).

## Introduction

Microsporidia are one of the most important group of protozoan pathogens that infect insects (Brooks, 1988; McLaughlin, 1971). They are found in most vertebrate and invertebrate phyla. They often cause chronic or debilitating disease in insects, which results in reduced fecundity and longevity, thereby presenting serious problems in the rearing of many insects, especially in commercial settings. Sub-lethal effects or reduced fitness is characteristic of most microsporidian infections in arthropods and has been documented in many insects (Gaugler and Brooks, 1975; Wilson 1974; Becnel and Geden, 1994; Geden *et al.*, 1995). They are also among the most common undetected pathogens in many insect cultures. Unlike other disease agents such as viruses, which have high virulence, or where there are more noticeable physical manifestations of the disease as in fungal infections, microsporidia may persist for several generations undetected without destroying the colony (Goodwin, 1984). Undetected disease organisms used in research can compromise physiological and basic biological studies, possibly leading to incorrect conclusions from such studies (Goodwin, 1984). It is therefore of primary importance that insects and other organisms are disease-free when they are used as experimental organisms. This is also very important in commercial production of arthropods that are used as biological control agents, because accidental

sale and release of diseased parasitoids and predators can compromise the success of the releases. Disease prevention strategies must therefore be in place to constantly monitor and screen for the presence of microsporidia, and once detected, remedial action must be implemented (Becnel and Andreadis, 1999).

A simple control strategy for eliminating microsporidian infection in an insect colony and one of the earliest methods was developed independently by Pasteur and Cantoni (Steinhaus, 1963) for eliminating pebrine disease caused by *Nosema bombycis* in silkworms, *Bombyx mori,* which was devastating the silkworm industry. After determining that the disease was transmitted transovarially, females were isolated for oviposition. After oviposition their infection status was determined and those that were infected were eliminated and the healthy ones pooled to found a new colony. The disadvantage of this technique is that it is labor-intensive and requires large numbers of healthy insects to ensure an adequate genetic base for the colony. The method is also only useful in situations where infection is less than 100%. However, this protocol combined with good sanitary practices remains the most effective approach to eliminate microsporidia from insect colonies (Boucias and Pendland, 1998). Good sanitation practices such as sterilization of equipment used to rear the insects can also be helpful in managing microsporidiosis in colonies (Hamm *et al.*, 1971; Undeen and Vavra, 1997). Cantwell and Shimuki (1969) were able to eliminate *Nosema* disease and increase production of honey from beehives by heat sterilization of equipment.

Various forms of heat therapy have been used successfully to eliminate microsporidian infection in insects. In those instances where it is effective this method exploits the high tolerance to heat of the host compared to the parasites (Becnel and

Andreadis, 1999). Raun (1961) successfully eliminated *Perezia* (=*Nosema*) *pyraustae* in the European corn borer, *Ostrinia nubialis* (Lepidoptera: Pyralidae), by heat-treating the eggs at 43°C for 30 minutes. Reduction in the infection of the microsporidian *Nosema partelli* in the stem borer *Chilo partellus* (Lepidoptera: Pyralidae) was reported by Kfir and Walters (1997) by holding infected eggs at 49°C for 20 minutes. Disease in *Muscidifurax raptor* caused by *Nosema muscidifuracis* was reduced by immersing infected eggs within the host puparia in a 47°C water bath for 30 – 45 minutes, although host survival was poor (Geden *et al.*, 1995). Rearing infected insects at an elevated temperature reduce microsporidian prevalence. This technique in combination with the Pasteur and Cantoni methods (Steinhaus, 1963) has been used to establish healthy colonies (Allen and Brunson, 1947; Raun, 1961; Hamm *et al*., 1971).

Many chemicals have been tested for activity against microsporidia since they were found to be the causative agent of pebrine disease, which was affecting the silkworm industry. Fumagillin, an antibiotic derived from the fungus *Aspergillus fumigatus* was one of the earliest compounds to be tested with some success. It reduced the infection caused by *Nosema apis* in honey bees (Katznelson and Jamieson, 1952; Bailey, 1953; Moffet *et. al*., 1969) and in the boll weevil, *Anthonomus grandis* (Flint *et al.,* 1972). However, fumagillin did not have any effect on the microsporidia *Pleistopora (=Endoreticulatus) schubergi* found in *Anaitis efformata* (Briese and Milner, 1986). It was also ineffective for the control of *Nosema muscidifuracis* in *Muscidifurax raptor* (Geden *et al*. 1995). The antibiotic rifampicin was found to decrease the levels of microsporidian infection when it was administered *per os* to *Encarsia* species infected with *Nosema* disease (Sheetz *et al.*, 1997).

Hsiao and Hsiao (1973) reported that incorporation of the fungicide benomyl in artificial diets of the alfalfa weevil eliminated *Nosema* parasites, without deleterious effect. However, Harvey and Gaudet (1977) reported that benomyl treatment resulted in reduction in fertility and egg hatching of the eastern spruce budworm, *Choristoneura fumiferana,* despite some reduction in microsporidian infection levels. Brooks *et al.* (1978) reported a resurgence of the microsporidian *Nosema heliothidis* in the corn earworm in the pupal and adult stages after treatment with benomyl, although it appeared to have reduced the infection in the larval stage.

Albendazole, a benzimidazole caused significant reduction in the number of infected cells *in vitro* in *Spodoptera frugiperda* cells, and also *in vivo* in *Helicoverpa zea* larvae and adults, but there was resurgence of the infection when the drug was withdrawn (Haque *et al.* 1993). This drug also has been used successfully to treat chronic diarrhea due to microsporidian infection in AIDS patients (Blanshard *et al.*, 1992; De Groote *et al.,* 1995; Joste *et al.,* 1996; Didier *et al.*, 1996).

The present chapter describes my evaluation of various physical and chemical methods for management of microsporidiosis caused by *Nosema muscidifuracis* in *Muscidifurax raptor*.

## Materials and Methods.

### Insect colony maintenance.

Parasitoid and host were reared and obtained from insect colonies maintained at the USDA's Center For Medical, Agricultural and Veterinary Entomology (Chapter 2), and except otherwise stated, all insects used in these experiments were obtained from these colonies.

Radiation Treatment.

Infected *M. raptor* females that had been mated (N=100) were given 1-2 day-old house fly pupae at a host-parasitoid ratio of 3:1 for 24 hours for oviposition. During this period most of the parasitoid eggs would not have hatched. The 300 pupae were then divided into 6 batches of 50 pupae each and placed in 30ml plastic cups and labeled appropriately. One set of fifty pupae was kept as a control. Each of the five remaining batches was then subjected to 20, 40, 60, 80, or 100 Grays of gamma radiation, respectively, in a cesium 127 radiation source. Twenty Grays represented the minimum dosage that we felt was practical with this irradiator. All the treatments were then kept at room temperature between 24 - 25 °C until emergence. After emergence (if any) the parasitoid progeny were sexed and scored for infection by making whole mount squashes of the insects and looking for microsporidian spores under a phase contrast microscope. The experiment was replicated two times. The above procedures were also used for the pupal stage of *M. raptor*. In this case, after *M. raptor* had oviposited in the house fly host, the pupae were held for about 12 days at 25°C to allow development to the pupal stage. They were then subjected to irradiation treatment as described above and then held until eclosion. Emerged adults were sexed and scored for infection as described above. The experiment was replicated two times.

Heat shock Treatment.

In these experiments 800 *M. raptor* females infected with *N. muscidifuracis* at a prevalence level greater than 97% were exposed to 3600 live house fly pupae for oviposition for 24 hours in groups of 200 females per 900 pupae to minimize

superparasitism. The exposure period of 24 hours ensured that only the eggs of the parasite was present. This stage was targeted because eggs were found to contain the fewest spores (Zchori-Fein *et al.,* 1992). After the 24-hour exposure period, the females were removed and the pupae mixed together. They were then divided again into 9 groups of 400 pupae for each treatment. Four groups of pupae were subjected to heat in an oven set at 40°C with a high humidity (70 –90 % RH), for 1, 3, 5 or 7 hours, respectively. Four other groups of pupae were subjected to heat in another oven set at 45°C (humidity, 70-90%RH) for 1, 3, 5, or 7 hours, respectively. A batch of 400 pupae serving as controls was held at room temperature of 24 –25°C. After the heat shock treatment the pupae were held at 24-26°C (RH, 70%) until progeny emerged. The parasitoids that emerged were counted, sexed and scored for infection.

In a similar test, four batches of 400 parasitized pupae were placed in an oven set at 50°C, again with RH of 70-90% for either 15, 30, 45, or 60 minutes. Another set of four batches was subjected to the same exposure times in an oven set at 47°C also with RH of 70 – 90%. Controls were held at 24 - 26°C RH 70%. The progeny that emerged were counted, sexed, and scored for infection as above. The experiment was replicated two more times.

The data were analyzed by G tests of independence comparing the emergence success and infection status of treated groups with the controls.

Continuous rearing at elevated temperature

House fly pupae that were 2 to 3 days old were weighed out into 6 groups of 1000 each and exposed to 250 infected *M. raptor* females (from the infected colony) per group for 24 – 30 hrs in 220 ml paper cans for oviposition. Parasitoids were removed from the

pupae after this period and the pupae were mixed together (for randomization) and again separated into three groups of 2000 pupae each. The infection level of the ovipositing females, which are designated as the parentals, was sampled for infection by making whole mount squashes and examining under a phase contrast microscope for spores. The infection level was recorded. In addition10 females were ground in 3 ml of deionized water in a glass tissue grinder and spore counts were made with a hemocytometer (Cantwell, 1970) to determine an initial spore load in the parasitoids. This was repeated three more times and the mean was recorded. The above procedures were repeated for males from the same cohort of insects as the parents used for oviposition.

Each paper can containing the pupae was placed in a rearing box (70 by 47 by 53 cm) with a trough of saturated sodium chloride solution to provide high humidity of about 70%. The cages were then placed in temperature-control walk–in chambers, set to run at 25, 30, or 32°C. Of the 2000 parasitized pupae set for each treatment group, 500 pupae were gel-capped and set aside at each temperature, to form the first generation (G-1). When they emerged a sample of 10 males and females were ground separately in a glass tissue grinder and spore counts were done as above. This was repeated for two additional samples of 10 parasitoids for both sexes. The infection level was also checked on a sample of at least 40 individual males and at least 50 females of the emerged parasitoids. The rest of the parasitoids that emerged were provided with host pupae at a constant host: parasitoid ratio of 20:1 to provide progeny for the next generation (G-2). When the next generation emerged the above procedures were again carried out up to the third generation (G-3).

The results were analyzed with the PROC GLM Procedure of Statistical Analysis System (SAS Institute, 1992) by evaluating the infection level and spore load concentration as a function of the rearing temperature. Means were separated by the method of Tukey.

Drug Treatment.

Incorporation of drug into host.

To incorporate the drugs in house fly host, about 25 mg of house fly larval diet, which had been mixed with water (5 parts diet to 4 parts water by volume) were weighed into a 30-ml plastic cup. The drug albendazole was weighed to constitute a 0.1, 1.0 or 10 % by weight of the fly diet and thoroughly mixed with the diet. House fly $2^{nd}$ instars (N=50) were placed in each concentration of diet. The cup was then covered with a cotton cloth and placed in a 25ºC chamber with relative humidity of 60 – 80%. The cups were checked daily for moisture and re- moistened as needed to prevent dehydration and held until pupation. The same procedures were repeated with rifampicin and the experiment was replicated for a total of 3 trials for each drug.

After pupation, the pupae were counted and weighed for each drug concentration. Two days after pupation, the pupae were exposed to infected *M. raptor* females at a host: parasite ratio of 5:1 for oviposition. After a period of 24-36 hours the parasitoids were removed and the pupae were held until emergence. Emerged parasitoids were counted and sexed, and their infection status was determined by making whole mount squashes of the parasitoids and checked under the microscope for spores.

Per os administration of drug.

In these experiments, 100 mg of each drug were mixed separately in a 30-ml plastic cup with 2g of honey, 200µl of glycerol and 0.5 ml of water to give a final drug solution of about 3% (Sheetz *et al.*, 1997). Preliminary bioassays showed that a 3% drug solution with honey and water was well tolerated by the parasitoids without adverse effect on survival. Female *M. raptor* (N = 400) that had been held for 12-24 hrs without food were placed in a 1-liter plastic container together with 200 males. A pipette was used to dispense 0.5ml of the drug solution which was placed on a 5 × 7 cm slide and was covered with Kimwipes® tissue paper. The slide was then placed inside the plastic container with the parasitoids for feeding. The sides of the containers were also streaked with the drug solution and the slide was replenished daily with the drug solution. The parasitoids were able to feed on the drugs through the tissue paper. After feeding for 24 hrs on the drug solution the parasitoids were given host pupae for oviposition for 24 hrs at 1, 3, 5, and 7 days after the initial exposure to the drug. The pupae were removed at the end of the 24-hr exposure and held at a temperature of 24-26°C for emergence. The emerged progeny from each successive oviposition interval were counted and scored for infection status as described previously. Mortality was assessed at 1, 3, 5, and 7 days post drug exposure. The experiment was replicated at least two more times for each drug. In another set of experiments a combination of both drugs in 1:1 ratio of 100 mg each was made and the procedures described above were carried out.

Mortality, emergence success, progeny production and infection rate were analyzed and evaluated with drug treatment as the dependent variable in the PROC GLM procedures of SAS (SAS Institute, 1992) and means separation method of Tukey.

Fitness studies of cured parasitoids

Establishing an uninfected colony of *M. raptor*

The Pasteur method was used to establish clean colonies from the treatment groups of 47°C and 50°C in the heat shock experiments and also from the drug experiments. A total of 150 female parasitoids that had been mated from these groups (treated at 45 and 60 minutes) were placed singly in 30-ml cups and numbered from 1 through 150. They were then supplied with 60 house fly pupae for a 3-day period of oviposition and feeding. After the third day each female was checked for infection. The pupae of those females that were infected were discarded and the uninfected females were pooled and held at temperature of 24 – 26°C for emergence. The progeny were again checked for infection to make sure that they were not infected. To establish clean colonies from the drug treatments, 50 female progeny from the fourth exposure (7 days post drug administration) were placed singly in 30ml cups and given 60 pupae each for 3 days. After the third day the females were assessed for infection status and the pupae that had been stung by infected individuals were discarded. Pupae stung by uninfected females were pooled for each drug and held until emergence. The emerged progeny were again assessed for infection. After successive observations of the infection status of the progeny for two generations demonstrated no resurgence of infection in the uninfected lines, they were pooled together to form one uninfected colony.

Parasitoid induced mortality, parasitism level, progeny production of infected and uninfected *M. raptor.*

After emergence, 12 groups each of 5 females and 2 males (1-2 day old) that were infected and another 12 groups each of 5 females and 2 males that were uninfected were placed in a 30ml cup and given fresh house fly pupae as host at a host: parasitoid ratio of 5:1 for 24 hrs. Each group was checked daily for 7 days and the dead were removed and recorded. Surviving insects were given fresh hosts at the host: parasitoid ratio of 5:1 and counted daily. Emerged house flies were counted, and the uneclosed pupae were held at 24 - 25°C for parasitoid emergence. Differences in parasitoid-induced mortality and production of male and female progeny as a function of infection status were evaluated by ANOVA, PROC GLM procedures of SAS (SAS Institute, 1992).

## Results

Radiation treatment:

The egg stage of *M. raptor* exposed to radiation from a Cs 127 source was very radiosensitive and doses tested were lethal. There was no emergence of adult parasitoids even from the lowest radiation dose of 20 Grays (Table 4.1). In contrast emergence from controls was about 60%. The pupal stage was more resistant to the radiation treatment compared to the egg stage. All treatments of the pupal stage had successful emergence to the adult stage, and varied from 87% - 114% of the controls. However none of the tested treatment doses had a therapeutic effect; infection was 100% in all groups.

Heat shock

Relative survival of *M. raptor* subjected to heat shock at 40°C decreased with time and was about 50% for an exposure time of 7 hours compared with the untreated controls. Treatment effects on infection were small and only significant at 3 and 5 hours. At 45°C survival was about 5% of the controls at an exposure period of 5 hours. Heat treatment for 7 hours was lethal at 45°C, and survival at 5-hour exposure was only about 5% compared with controls (Table 4-2). The reduced survival was significant for all temperatures except at 40°C for the treatment of 1 hour (Table 4-2). Pronounced curing (90-100%) was obtained at 45°C for 3 and 5 hours, although the survival was very low at 5 hours. Significant reductions in the infection rate were observed at all exposure times for the exposure conducted at 47°C and 50°C (Table 4.3). Over 85% reduction in infection was observed for exposure times of 60 and 75 minutes at 47°C and survival was 58-71% compared with controls. There was 100% cure (0% infection) at 50°C when the pupae where exposed for 45 and 60 minutes, with relative survival of 18 and 8%, respectively (Table 4-3). No parasitoid emerged at 50 °C when pupae were exposed for 75 minutes.

Rearing at elevated temperature.

Infection level of the emerged parasitoids for both sexes for the first generation at 30°C was 100% and was not different from those reared at 25°C (Table 4-4). However there were small, but statistically significant, reductions of the infection rates (7 and 4.2 % for females and males, respectively) in the 1$^{st}$ generation when reared at 32 °C (Table 4-4). In the second generation, there was a similar trend in that the parasitoids reared at 30°C did not show any reduction in the infection level for both sexes when compared to those reared

at 25°C. At 32°C the infection levels for females were not different from the 25°C controls, but the infection levels of males were lower (10%) than in the controls. There were no differences in the infection levels for the 3rd generation in both sexes.

Spore concentrations were higher for females overall compared with the males (Table 4-5), and it decreased with successive generation for the males, while the females shows some increase in the second generation at temperatures of 25 and 30°C. There was a decrease in the spore load concentration within a generation as temperature increased for both sexes generation. The greatest differences in spore concentration between the first and third generations were 70 and 61% for the female and male respectively at 32°C. The reduction in spore concentration between the first and third generation was about 51% for both sexes at 30°C. The lowest reduction in spores between the first and the third generation was 14.3 and 4.6% respectively for females and males at 25°C. Parasitoids reared at 32°C had about one third as many spores as the 25°C controls after a single generation. Rearing at 32°C for 3 generations resulted in a 7-8-fold reduction in spore concentration compared with the controls.

Incorporation of drug in larval diet of host.

Both albendazole and rifampicin showed strong effects on the immature stages of the house fly. There were pronounced differences in the pupal size (Fig 4-1). Mortality increased in the larvae that were fed diet with albendazole, which resulted in reduced pupation success and pupal weights at all doses tested (Table 4.6). Rifampicin also showed a drastic reduction in the pupal weight and many of them were hollow and light in color indicative of less tanning (Fig 4.1). Parasitoids that emerged from all concentrations of the albendazole groups were still infected (Not shown). The pupae from the rifampicin treated

group were mostly hollow and very small in size relative to the controls, and were not exposed to host.

Per os drug treatment of adult parasitoids.

Mortality

Treatment of adult parasitoids with each drug showed no significant difference in the mortality up to day 5 post treatment (Table 4-7). At day 7 the mortality for the rifampicin treated group was higher (72.5%) than for the controls (54.4%) and the albendazole treated parasitoids (53.8%). More than 50% of all the parasitoids were dead by day 7 in all groups.

Emergence

The number of parasitoid progeny produced decreased with each exposure time. Rifampicin treated parentals produced the least progeny at all exposure periods and was almost half as much as the controls for the first exposure period (Table 4-8). By day 7 the progeny production was 7.1, 10.6, 19.1, and 10.5% of the progeny at day 1, respectively, for the controls, albendazole, mixture and rifampicin. The mixture produced the most progeny at day 7, despite the fact that they did not record the lowest mortality (Table 4-7).

Infection level

All the drug treatments resulted in significant reductions of infection by day 3 and therefore was partially effective in reducing maternal transmission of the parasite, especially on day 7 (Table 4-9). Rifampicin was the most effective treatment. It caused a reduction of almost 40%, while treatment with albendazole and the mixture reduced infection by about 20%. All the drug treatments produced similar infection levels of about 98% for the first day post treatment, and this was not different from the controls, in which

all the progeny were infected. There was a slight reduction in the infection level of progeny at day 3 from the controls, ranging from about 6% for the mixture to about 3% for the rifampicin and albendazole treatments. This reduction was significantly different from the controls, but there were no differences among the drugs treatments (Table 4-9). The percentage of females produced increased slightly with time (Fig. 4-3). Rifampicin showed the greatest percentage increase in females with about 65% on day 7, up from about 40% on day 1. This corresponds to the highest reduction of infection (Table 4-9).

Fitness studies

There were no differences in the survival of the females for the first five days of observation. However, differences were observed on day 6 and 7 (Table 4-10). By day 7 only 16% of the infected females were surviving while 60% of the cured females were alive. No difference in the survival was observed with the males, although a greater proportion of the cured males were surviving compared to the infected males on each day, except for days 3 and 4 were they were equal. By day 7, 50 % of the cured parasitoids were alive compared to only 28% for the infected ones (Table 4-10).

Parasitoid induced mortality (PIM), which is a measure of the attack rate and parasitism, were higher for the cured (uninfected) parasitoids and ranged from 1.1 to 12 times higher than the infected parasitoids (Table 4-11). The cured parasitoids also showed a higher percentage of females compared to the infected parasitoids, and produced almost twice as many females per day per female as the infected parasitoids (Fig 4-2). This was significant at all days (Table 4-12). However, production of male progeny by uninfected parasitoids was similar to that of the infected group, except for days 1, 6 and 7 in which there were significant differences (Table 4-12).

## Discussions

*Nosema muscidifuracis* in *M. raptor* is maintained by a transmission cycle that involves both transovarial and horizontal transmission. The pathogen is acquired horizontally when uninfected immatures cannibalize infected immatures in superparasitized puparia and also when uninfected adults feed on infected parasitoid immatures within host puparia. Vertical transmission occurs from mother to offspring via the infected eggs (Geden *et al.*, 1995). Microsporidian development can be influenced by environmental factors such as temperature and host nutritional sources and levels (Becnel and Undeen, 1992). Also elevated temperature can inhibit or reduce the growth of microsporidia in insects (Wilson and Sohi, 1977). Our strategy in attempting to manage the disease was to kill the vegetative stages or inactivate the spores in the egg stage so that the resulting progeny would be free of the disease. The spore load in the egg stage and other immature stages are usually low in comparison to the adult (Zchori-Fein *et al.*, 1992). Several reports (Kelly and Anthony, 1979; Wilson, 1974; Teetor and Kramer, 1977; and Baribeau and Burkhardt, 1970) have shown that sunlight or ultraviolet radiation can inactivate microsporidian spores, and there is at least one report (Undeen *et al.,* (1984) in which spore viability measured by both infection rate and intensity of infection in *Nosema algerae* was lost when purified spores were subjected to gamma radiation. In these experiments gamma radiation treatments were lethal to the parasitoid eggs and were ineffective in causing any curing effect in the pupal stage at the doses tested (Table 4-1).

*Nosema muscidifuracis* appears to be very resilient to heat therapy. The pathogen was able to survive temperature extremes as high as 50°C for 60 minutes (Table 4-3). However, it is difficult to ascertain the exact temperature the parasitoid and pathogen

actually experienced in these tests, because it may have taken some time for the host puparia to reach the desired temperature. Exposure of *M. raptor* at 50°C for 45 minutes was very effective in curing it from *Nosema* disease, resulting in 100% cure (Table 4-3). This exposure temperature gave a relative survival of 18% which seems to be an acceptable level of mortality. Therefore this method could be adopted in salvaging colonies that are 100% infected. Other temperatures and exposure times were also effective in reducing the infection level with less parasitoid mortality, for example, an exposure time of 3 hours at 45°C resulted in about 90% cure with a relative survival of almost 50%. The Pasteur method could then be used to establish a healthy colony. The results obtained here were an improvement of those obtained by Geden *et al.,* (1995). In their experiments they submerged infected *M. raptor* eggs within host puparia in water baths at various temperatures in an effort to transfer heat to target tissues more rapidly. Although exposure of infected *M. raptor* eggs in a 47°C water bath for 45 –60 minutes greatly suppressed *N. muscidifuracis*, the treatment resulted in poor parasitoid survival. Submerging the parasitoid in water may have limited exposure times tolerated by the immature parasitoid because of asphyxiation and may have resulted in the lower survival.

There were slight reductions in the infection level when infected parasitoids were reared at continuous elevated temperature of 30 and 32°C for up to three generations (Table 4-4). These however, were not significant for the females but were significant for the males at 32°C for the first and second generation. Although rearing at elevated temperatures had only modest effects on infection levels, it did have substantial effects on the spore concentration. It resulted in a reduction of the numbers of spores, albeit not to a sufficient degree to effect cure (Table 4-5). In our experiment we only reared the parasitoids for up

to three generations. It is possible that rearing for additional generations may have had therapeutic value. Wilson (1979) also observed a reduction in the spore production of *Nosema fumiferanae* in the spruce budworm when colonies were reared continuously at 30°C. Nevertheless, there was resurgence of the infection when the hosts were returned to lower temperatures. It is possible that since the spores are generally resistant to heat, they may have survived, but were inhibited at the higher temperature, and when they were moved to lower temperatures the spores were able to germinate and resume the infection. However, Wilson and Sohi (1977) were able to completely eliminate the microsporidia *Nosema disstriae* from *Malacosoma disstria* when cultures were reared at 35°C for 28 days.

There has been renewed interest in microsporidia research in recent years, probably due to awareness of their importance as enteric pathogens in immunocompromised patients with HIV infection or patients treated with immunosuppressive drugs (Weiss, 2001; Costa and Weiss, 2000). Consequently many compounds are currently being tested for antimicrosporidial activity.

Albendazole is a benzimidazole that has been used extensively and has shown some promise in curbing microsporidia infections. It is a microtubule inhibitor and is effective against *Encephalitozoon* species that infect humans and other mammals (Didier *et al.*, 2000). However in our tests with albendazole in which we attempted to incorporate the drug in the diet of the house fly host, it was ineffective in reducing the infection and was somewhat detrimental to the pupae, resulting in low number of pupae and low pupal weight (Table 4.6). The antibiotic rifampicin was also ineffective when used in this manner and was detrimental to the larvae. Although many of the larvae were able to pupate, the

resulting pupae were small, hollow and non-viable. The antibiotic nature of rifampicin might have killed many of the microorganisms that the larval host uses for nutrition, thereby denying the larvae an essential source of nutrients.

*Per os* administration of either albendazole, rifampicin or a mixture of both drugs gave some cure ranging from about 20% for albendazole and the mixture to about 40% with rifampicin during the fourth exposure to host pupae for oviposition. The first and second exposure periods showed a slight reduction in the infection level, but this was not significant. Rifampicin was the most effective of the treatments (Table 4-9). Rifampicin-treated parasitoids have the highest percentage of females of any treatment and this corresponded to the lowest infection level. The presence of microsporidia seems to distort the sex ratio in *M. raptor* towards the males (Zchori-Fein *et al* 1992; Geden *et al.* 1995). Rifampicin was also used successfully in eliminating a *Nosema* pathogen from an *Encarsia* parasitoid without any evidence of resurgence (Sheetz *et al.*, 1997). In many cases after treatment with chemicals for controlling microsporidia, there is resurgence of the parasite once the hosts are taken off the drug (Briese and Milner, 1986; Lynch and Lewis, 1971).

A primary concern in subjecting *M. raptor* to heat shock and also rearing them at elevated temperature near their upper limit (Chapter 3) was their fitness after elimination of the disease. Our results show that the cured parasitoids were substantially more productive in terms of life history traits than the infected parasitoids (Tables 4-10 –12). The cured parasitoids produced almost twice as many females per day than the infected females (Fig. 4-3). Although there were no significant differences in the survival for the first 5 days for the females (Table 4-10), there were significant differences in the progeny production (Table 4-12), dispelling speculation that differences in the progeny production might be

due to differences in mortality. The males showed no differences in the survival between infected and cured. It appears that the infection is more severe in the females than in the male. This could be supported by the fact there were more spores in the females than in the males (Table 4-5). Whereas the infection in the females ensures infection of its progeny by transovarial transmission, the role of the infection in the males is not known and appears to be a dead end, because there is no evidence of paternal or venereal transmission of the pathogen. However spores have been observed in the meconium of immatures in both male and females and also in the feces of adult males. These environmental spores may be involved in horizontal transmission and infection of new hosts. A healthy colony has been established from the cured parasitoids and there is no evidence of resurgence in it after more than 10 generations in culture.

In summary our results indicate that *Nosema* diseases in *M. raptor* can be eliminated from colonies of *M. raptor* using simple heat treatment or drug therapy methods followed by isolation and combination of clean family lines, and that such therapy results in greatly enhanced fitness of the parasitoids. These methods can be used easily by commercial insectaries or researchers with an interest in parasitoid biology.

Table. 4-1 Emergence and infection level of adult *M. raptor* after immature stages were treated with different doses of radiation.

| Dose / Stage | Number emerged | | | Percent infection |
|---|---|---|---|---|
| Grays | Female | Male | N | |
| 0 (Control) Egg | 12.5 | 18.0 | 50 | 100 |
| 20 | 0 | 0 | - | - |
| 40 | 0 | 0 | - | - |
| 60 | 0 | 0 | - | - |
| 80 | 0 | 0 | - | - |
| 100 | 0 | 0 | - | - |
| 0 (Control) Pupae | 16.5 | 18.0 | 60 | 100 |
| 20 | 16.0 | 23.5 | 60 | 100 |
| 40 | 105 | 24.0 | 50 | 100 |
| 60 | 18.0 | 20.0 | 55 | 100 |
| 80 | 14.5 | 21.5 | 50 | 100 |
| 100 | 11.5 | 18.5 | 40 | 100 |

Table 4-2. Relative survival and infection level of *M. raptor* (egg stage within host puparia) when subjected to heat at 40 or 45°C.

| Temp °C | Time (hrs) | Mean (SE) Emergence | Relative Survival[1] | $\chi^2$ | N | % Infection | $\chi^2$ |
|---|---|---|---|---|---|---|---|
| 40 | 1 | 199.5 (6.4) | 93.4 | 0.6ns[2] | 75 | 96.0 | 2.5ns[2] |
| 40 | 3 | 172.0 (17.0) | 80.6 | 6.0* | 117 | 93.3 | 8.5** |
| 40 | 5 | 157.0 (50.9) | 73.5 | 11.8** | 120 | 93.3 | 7.0** |
| 40 | 7 | 106.0 (12.0) | 49.9 | 53.1** | 90 | 96.7 | 1.9ns |
| 45 | 1 | 166.5 (13.4) | 78.0 | 7.9** | 68 | 88.2 | 12.2** |
| 45 | 3 | 101.5 (9.2) | 47.5 | 58.9** | 108 | 10.2 | 239.7** |
| 45 | 5 | 11.5 ( 6.4) | 5.4 | 362.1** | 23 | 0.0 | 117.2** |
| 45 | 7 | 0.0 | 0.0 | 490.1** | - | - | - |
| Control | | 213.5 ( 2.8) | 100.0 | - | 130 | 99.2 | - |

[1] Emergence success as a percent of control emergence.

[2] ns, P>0.05; *, P<0.05; ** P<0.01 (G-tests of independence comparing emergence success and infection status of treated groups with controls)

Table 4-3. Relative survival and infection level of *M. raptor* (egg stage within host puparia) when subjected to heat at 47 or 50°C.

| Temp °C | Time (min) | Mean (SE) Emergence | Relative Survival[1] | $\chi^2$ | N | % Infection | $\chi^2$ |
|---|---|---|---|---|---|---|---|
| 47 | 15 | 184 (45.3) | 96.6 | 0.2ns | 118 | 83.9 | 11.5** |
| 47 | 30 | 170 (46.7) | 89.2 | 1.6ns | 119 | 73.9 | 25.6** |
| 47 | 45 | 135 (33.9) | 70.9 | 13.5** | 229 | 35.8 | 127.8** |
| 47 | 60 | 120 (26.2) | 71.1 | 23.3** | 198 | 14.6 | 214.1 ** |
| 47 | 75 | 130 (47.4) | 57.5 | 16.4** | 158 | 12.7 | 208.4** |
| Control | | 190 (38.9) | 100.0 | - | 100 | 97.0 | - |
| 50 | 15 | 123.0 (49.0) | 57.6 | 34.9** | 166 | 84.3 | 54.5** |
| 50 | 30 | 106.0 (48.0) | 49.6 | 53.1** | 129 | 65.1 | 7.9** |
| 50 | 45 | 38.5 (16.3) | 18.0 | 209.3** | 40 | 0.0 | 43.7** |
| 50 | 60 | 17.5 (3.5) | 8.2 | 317.6** | 30 | 0.0 | 32.6** |
| 50 | 75 | 0.0 | 0.0 | 490.0** | - | | - |
| Control | | 213.5 ( 2.8) | 100.0 | - | 130 | 99.2 | - |

[1] Emergence success as a percent of control emergence.

[2] ns, P>0.05; *, P<0.05; ** P<0.01 (G-tests of independence comparing emergence success and infection status of treated groups with controls)

Table. 4-4 Infection level [mean (SE)] of *M. raptor* reared at various constant temperatures through three generations.

| Temp °C | Sex | Generation 1 | 2 | 3 |
|---|---|---|---|---|
| 25 | Females | 100.0 (0.0) a | 100.0 (0.0) a | 100.0 (0.0) a |
| 30 | | 100.0 (0.0) a | 100.0 (0.0) a | 96.4 (2.3) a |
| 32 | | 93.0 (2.1) a | 98.0 (1.7) a | 96.3 (2.2) a |
| ANOVA F[1] | | 7.78 * | 1.43 ns | 0.86 ns |
| 25 | Males | 100.0 (0.0) a | 100.0 (0.0) a | 98.4 (1.5) a |
| 30 | | 100.0 (0.0) a | 100.0 (0.0) a | 100.0 (0.0) a |
| 32 | | 95.8 (1.1) b | 90.0 (0.0) b | 97.5 (2.0) a |
| ANOVA F[1] | | 34.04 ** | Infinity ** | 0.54 ns |

[1] $P > 0.05$, ns; $*P \leq 0.05$; $** P \leq 0.01$

Means followed by the same letter within a column for each sex are not statistically different at $P = 0.05$ using Tukey's means separation method.

Table 4-5. Spore concentration [mean × $10^6$ (SE)] of 3 groups of 10 *M. raptor* infected with *N. muscidifuracis* reared at various temperatures through three generations.

| Temp °C | Sex | Generation | | |
|---|---|---|---|---|
| | | 1 | 2 | 3 |
| 25 | Females | 13.75 (0.5) a | 15.08 (0.5) a | 13.11 (0.49)a |
| 30 | | 7.25 (0.26) b | 7.55 (0.58) b | 3.53 (0.39) b |
| 32 | | 4.76 (0.71) c | 3.68 (0.19) c | 1.85 (0.32) b |
| ANOVA F[1] | | 77.73 ** | 162.45 ** | 222.18 ** |
| 25 | Males | 10.33(0.47) a | 9.23 (0.37) a | 8.85 (0.22) a |
| 30 | | 5.63 (0.34) b | 4.60 (0.58) b | 2.73 (0.39) b |
| 32 | | 3.33 (0.09) c | 2.45 (0.10) c | 0.98 (0.11) c |
| ANOVA F[1] | | 112.44 ** | 79.44 ** | 232.72 ** |

[1] $P > 0.05$, ns; *$P \leq 0.05$; ** $P \leq 0.01$; df = (2, 6)

Means followed by the same letter within a column for each sex are not statistically different at P= 0.05 using Tukey's means separation method.

Table 4-6. The effect of various concentrations of albendazole on the pupal weight of *Musca domestica*. Four groups of 50 larvae fed on a diet containing various concentrations of albendazole until pupation.

| Percent Concentration of drug | N | Mean number of pupae (SE) | Mean wt (SE) of pupae(mg) |
|---|---|---|---|
| Control | 183 | 45.8 (1.0) a | 16.23 (0.5) a |
| 0.1 | 136 | 34.0 (3.1) b | 10.6 (0.9) b |
| 1 | 146 | 36.5 (2.6) b | 10.3 (1.0) b |
| 10 | 148 | 37.0 (1.0) a,b | 9.6 (0.9) b |
| ANOVA F[a] | | 5.75* | 13.17** |

[a] $P > 0.05$, ns; *$P \leq 0.05$; ** $P \leq 0.01$; df = (3, 12)

Means followed by the same letter within a column are not statistically different at P= 0.05 using Tukey's means separation method.

Table 4-7 Mean percent mortality (SE) of *M. raptor* after *per os* drug treatment at different exposure period.

| Type of[a] Treatment | Exposure of host post- drug treatment in days | | | |
|---|---|---|---|---|
| | 1 | 3 | 5 | 7 |
| Control | 6.9 (1.7) a | 23.3 (1.8) a | 36.6 (2.2) a | 54.4 (1.1) b |
| Albendazole | 5.5 (2.3) a | 19.0 (5.2) a | 31.2 (6.3) a | 53.8 (5.9) b |
| Mixture | 8.6 (0.4) a | 25.3 (2.0) a | 40.1 (2.1) a | 61.7 (1.4) a, b |
| Rifampicin | 12.7 (3.0) a | 38.9 (11.2) a | 54.8 (10.2)a | 72.5 (1.4) a |
| ANOVA F[b] | 2.19 ns | 1.86 ns | 2.69 ns | 5.51 * |

[a] 3% of drug solution.
[b] $P > 0.05$, ns; * $P \leq 0.05$; $P \leq 0.01$. df = 3
Means with the same letter within a column are not significantly different at P =0.05 using Tukey's means separation method.

Table 4-8. Emergence success of progeny [mean (SE)] of *M. raptor* emerging at a range of exposure periods after *per os* drug treatment.

| Type of[a] Treatment | Time of exposure of host post-drug treatment in days: 1 | 3 | 5 | 7 |
|---|---|---|---|---|
| Control | 1026.3 (82.2)a | 671.3 (32.8)a | 518.0 (81.7) a | 73.0 (2.6)a |
| Albendazole | 923.7 (46.5)a,b | 776.7 (83.3)a | 365.0 (106.5) a | 98.0 (49.2)a |
| Mixture | 937.3 (107.7)a,b | 652.3 (43.7)a | 315.4 (93.2) a | 179.0 (55.0)a |
| Rifampicin | 557.3 (128.4)b | 423.8 (119.1)a | 150.0 (50.6) a | 59.3 (34.3)a |
| ANOVA F[b] | 4.66 * | 0.76 ns | 3.14 ns | 1.85 ns |

[a] 3% of drug solution.
[b] P>0.05, ns; *P ≤0.05; ** P ≤0.01; df = (3, 8)
Means followed by the same letter within a column are not statistically different at P=0.05 using Tukey's means separation method.

Table 4-9. Percent infection rate of progeny [mean (SE)] of *M. raptor* emerging at various exposure periods after drug treatment.

| Type of [a] Treatment | Exposure of host post- drug treatment in days | | | |
|---|---|---|---|---|
| | 1 | 3 | 5 | 7 |
| Control | 100.0 (0.0) a | 100.0 (0.0) a | 100.0 (0.0) a | 99.1 (1.0) a |
| Albendazole | 98.6 (0.8) a | 97.1 (0.7) b | 91.5 (3.4) b | 78.6 (8.2) b,c |
| Rifampicin | 98.5 (0.8) a | 97.3 (1.0) b | 73.5 (6.7) c | 57.7 (4.4) c |
| Mixture | 98.9 (1.1) a | 93.4 (1.9) b | 86.5 (3.9) b,c | 81.7 (1.6) b |
| ANOVA F[b] | 1.1 ns | 10.24 ** | 15.55 ** | 22.96 ** |

[a] 3% of drug solution.
[b] P> 0.05, ns; * P ≤0.05; P ≤0.01; df=3.
Means with the same letter within a column are not significantly different at P =0.05 using Tukey's means separation method.

Table 4-10. Comparison of mean male and female survival of infected and uninfected *M. raptor* that have been cured of *Nosema muscidifuracis*. Twelve groups of 5 females and 2 males were observed for the first 7 days at holding temperatures of 24-26°C.

| Day | Sex | Mean survival (SE) | | | |
|---|---|---|---|---|---|
| | | Uninfected | Infected | F[a] | df |
| 1 | Female | 5.0 (0.0) | 4.9 (0.1) | 1.0 ns | 1,22 |
| 2 | | 4.9 (0.1) | 4.9 (0.1) | 0.0 ns | 1,22 |
| 3 | | 4.7 (0.1) | 4.8 (0.1) | 0.19 ns | 1,22 |
| 4 | | 4.6 (0.1) | 4.7 (0.1) | 0.16 ns | 1,22 |
| 5 | | 4.6 (0.2) | 4.3 (0.3) | 0.85 ns | 1,22 |
| 6 | | 4.2 (0.3) | 2.8 (0.4) | 6.64* | 1,22 |
| 7 | | 3.0 (0.5) | 0.8 (0.3) | 20.61** | 1,10 |
| 1 | Male | 2.0 (0.0) | 1.9 (0.1) | 1.0 ns | 1,22 |
| 2 | | 2.0 (0.0) | 1.8 (0.1) | 2.2 ns | 1,22 |
| 3 | | 1.8 (0.1) | 1.8 (0.1) | 0.23 ns | 1,22 |
| 4 | | 1.8 (0.1) | 1.8 (0.1) | 0.23 ns | 1,22 |
| 5 | | 1.8 (0.1) | 1.4 (0.2) | 3.48 ns | 1,22 |
| 6 | | 1.8 (0.2) | 1.3 (0.3) | 3.77 ns | 1,22 |
| 7 | | 1.0 (0.2) | 0.5 (0.3) | 1.36 ns | 1,10 |

[a] $P>0.05$, ns; $*P \leq 0.05$; $**$ $P \leq 0.01$.

Table 4-11. Comparison of parasitoid induced mortality (uneclosed pupae) of infected and uninfected *M. raptor* that have been cured of *Nosema muscidifuracis*. Twelve groups of 5 females with 2 males were given hosts at a host: parasitoid ratio of 20:1.

| | Parasitoid Induced Mortality (SE) | | | |
|---|---|---|---|---|
| Day | Uninfected | Infected | F[a] | df |
| 1 | 61.2 (2.9) | 43.9 (2.9) | 16.92** | 1, 16 |
| 2 | 79.4 (1.5) | 72.2 (4.9) | 1.18ns | 1, 14 |
| 3 | 85.0 (3.2) | 69.6 (4.5) | 5.09* | 1, 16 |
| 4 | 60.1 (5.6) | 49.8 (5.5) | 1.45 ns | 1, 16 |
| 5 | 47.4 (4.1) | 30.8 (4.2) | 8.54* | 1, 16 |
| 6 | 35.6 (3.6) | 10.9 (2.7) | 30.64** | 1, 20 |
| 7 | 28.8 (9.6) | 2.3 (1.2) | 7.56 * | 1, 10 |

[a] ns, $P > 0.05$; *$P \leq 0.05$; ** $P \leq 0.01$

Table 4-12. Comparison of mean progeny produced from infected and uninfected *M. raptor* that have been cured of *Nosema muscidifuracis* disease. Twelve groups of 5 females and 2 males were given hosts at a host: parasitoid ratio of 20:1 for 7 days.

| Day | Sex | Mean female progeny (SE) | | | |
|---|---|---|---|---|---|
| | | Uninfected | Infected | F[a] | df |
| 1 | Females | 23.2 (1.7) | 11.0 (1.6) | 26.06 ** | 1, 22 |
| 2 | | 52.7 (2.9) | 25.6 (2.3) | 53.45 ** | 1, 22 |
| 3 | | 44.4 (2.9) | 26.0 (2.7) | 21.45 ** | 1, 22 |
| 4 | | 31.9 (3.5) | 13.3 (2.6) | 18.37 ** | 1, 22 |
| 5 | | 24.7 (3.4) | 6.9 (1.6) | 21.96 ** | 1, 22 |
| 6 | | 13.3 (1.5) | 2.4 (0.9) | 39.57 ** | 1, 22 |
| 7 | | 11.0 (4.4) | 0.0 (0.0) | 6.36 * | 1, 10 |
| 1 | Males | 18.2 (1.6) | 13.5 (1.4) | 4.66* | 1, 22 |
| 2 | | 15.9 (1.5) | 17.7 (1.5) | 0.62 ns | 1, 22 |
| 3 | | 12.3 (1.4) | 17.0 (2.2) | 3.14 ns | 1, 22 |
| 4 | | 7.3 (1.5) | 6.9 (2.7) | 0.04 ns | 1, 22 |
| 5 | | 7.5 (1.5) | 4.9 (1.9) | 1.12 ns | 1, 22 |
| 6 | | 5.8 (0.8) | 1.4 (0.7) | 18.34** | 1, 22 |
| 7 | | 3.2 (1.1) | 0.0 (0.0) | 8.64* | 1, 10 |

[a]P>0.05, ns; *P ≤0.05; ** P ≤0.01.


Fig.4.1 Pupae of *Musca domestica* after 2nd instars were fed on 1% drug; a = rifampicin, b = albendazole and c = controls

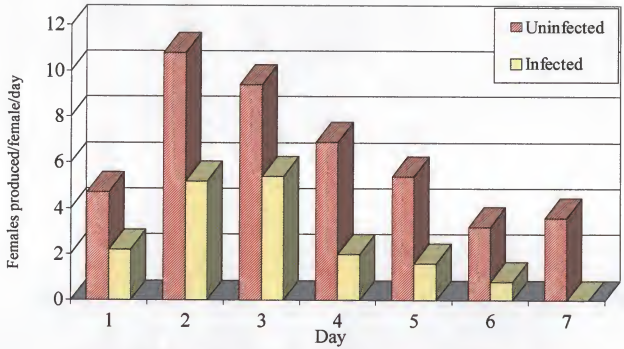



Fig 4.2. Female progeny production of infected and uninfected *M. raptor* that was cured of *Nosema muscidifuracis*.

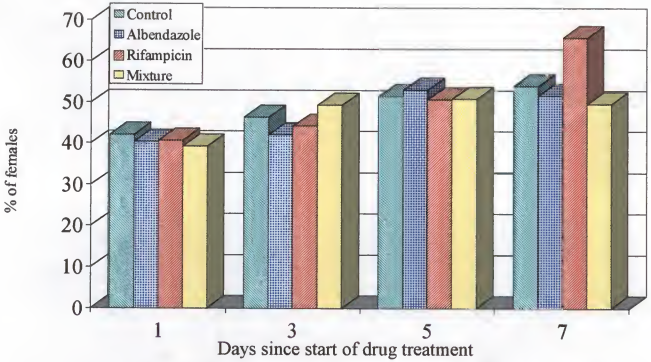



Fig. 4.3 Percentage of female progeny of *M. raptor* infected with *N. muscidifuracis* at different exposure times following drug treatment of parents.

# CHAPTER 5
# SUMMARY AND CONCLUSIONS

The successful implementation of biological control programs requires the production and release of healthy and vigorous natural enemies. *Muscidifurax raptor* is a pupal parasitoid of house flies and other muscoid flies, and has been used as a natural enemy in biological control programs to control muscoid flies associated with livestock and poultry production. It is one of the most abundant pupal parasitoids of house flies and is nearly cosmopolitan in distribution. These parasitoids have been found to be infected with the microsporidian pathogen *Nosema muscidifuracis*, which causes a chronic disease in adult parasitoids, resulting in reduced fecundity, searching ability, and reduced longevity. This reduced fitness severely hampers the use of this parasitoid in biological control programs. The pathogen infects the midgut epithelium, Malpighian tubules, ovaries, and fat body of both larvae and adult. The disease has been detected in virtually every colony of *Muscidifurax* species in the world, including those maintained in commercial insectaries. It is therefore pertinent that rigorous quality control procedures are in place to ensure that infected parasitoids are not released in the field.

Studies designed to find the host range of *Nosema muscidifuracis* showed that this microsporidium has a high propensity to germinate in a wide range of host. It causes infection in the *Spalangia* species, but the infection was light with low number of spores compared to those produced in *Muscidifurax raptor*. Despite the fact that *Nosema muscidifuracis* infected *Spalangia gemina, S. cameroni, S. endius, S. nigroaenea*, and

*Muscidifurax raptorellus*, transovarial transmission of the pathogen was limited to only *Spalangia gemina* and *M. raptorellus*. The disease and its effects on *M. raptorellus*, is probably similar to those found in *M. raptor* where it detrimentally affects its fitness. *Nosema muscidifuracis* failed to infect the house fly and the black dump fly, *Hydrotaea aenescens*. It is still possible that some Dipteran hosts may be susceptible to *N. muscidifuracis* from which *M. raptor* may also pick up the infection when it parasitizes the host.

Microsporidian infections are generally difficult to manage because of their intracellular parasitic lifestyle and also the highly resistant nature of the spore wall, which has chitinous inner layers and an outer proteinaceous layer (Colbourn *et al.*, 1994). The primary focus of this dissertation was to devise practical control strategies for managing infection of microsporidia in *Muscidifuracis raptor* by exploiting the parasitoid's biology. One of the parameters that was exploited was the developmental time. Infected parasitoids took longer to develop than healthy insects. The parasitoids were reared at various temperatures in order to find a temperature that would provide a high resolution in which infected and uninfected parasitoids can be collected with certainty. In evaluating this strategy, results show that large proportions of healthy females can be collected by retaining only those females that emerged in the first 24 hours of emergence at 25°C or during the first 48 hours of female emergence at 20°C. This is very useful because, even though infected males may emerge and overlap with the healthy females, previous experiments have proved that there is no paternal transmission of the pathogen. In employing heat to manage *N. muscidifuracis*, our strategy was to kill the vegetative stages or inactivate the spore stages so the emerging progeny would be healthy. *M.*

*raptor* was relatively more resilient to heat than other parasitoids tested, especially *Spalangia* spp. *and Tachinaephagus zealandicus* (Unpublished personal data). Exposure of infected *M. raptor* at 50°C. for 45 minutes resulted in 100% cure and a survival of 18%. Heat shock therapy was very effective in reducing the infections levels in infected *M. raptor*. Continuous rearing of infected *M. raptor* at elevated temperatures was not effective in reducing infection levels, even though there was substantial reduction in the spore concentrations. Radiation treatments were ineffective in reducing or eliminating infection in *M. raptor*.

Attempts to incorporate the drug, albendazole in the diet of house fly resulted in pupae with low weight. These pupae were not effective in reducing infection in *M. raptor* when they were used as host for infected *M. raptor*. The drug rifampicin incorporated into the diet of the house fly did not support normal development of the larvae, with the resulting pupae being non-viable. However, a 3% solution of these drugs mixed with honey and water resulted in a 20% reduction of the infection in adults for albendazole and 40% reduction in infection for rifampicin. A combination of both drugs did not produce any synergistic effect, but resulted in a 20% reduction in infection levels in adults. A healthy colony established from the infected colony based on these strategies showed a higher fitness in terms of fertility, longevity and attack rate than the infected colony. More importantly, this uninfected colony has not exhibited any resurgence of the infection and has been in culture for more than 10 generations. The results show that *Nosema* disease in *M. raptor* can be eliminated from colonies of *M. raptor* by simple heat treatment or drug therapy methods, followed by isolation and combination of a clean family line to establish uninfected cultures of *M. raptor*. Commercial insectaries or

researchers can easily adopt these methods. The healthy parasitoids have a greatly enhanced fitness with respect to its searching ability, fecundity, longevity, and dispersal. This strengthens its proven potential as a biological control agent for muscoid flies. Implementation of bio-based environmentally friendly control programs can substantially reduced the dependency on chemical pesticides usage and its ramifications, while helping to promote a sustainable, profitable livestock production industry and at the same time fostering a safe food supply (CJG, Unpublished).

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## BIOGRAPHICAL SKETCH

Carl Kenneth Kwame Boohene was born in Ghana and spent the early part of his childhood in London and Sierra Leone. He attended the University of Ghana in Legon, Ghana, where he obtained a B.Sc (Hons) degree in zoology. After graduation he worked as a teaching assistant in the Zoology Department in invertebrate zoology and genetics. He was later seconded to the Ghana Atomic Energy Commission to work on an International Atomic Energy Agency project on the control of tsetse using sterile insect techniques, where he was involved in the mass rearing of tsetse using in vitro and in vivo techniques. He came to the United States of America in 1993 and pursued a masters in forensic entomology from the University of Hawaii. He joined the Mosquito and Fly Research Unit of USDA- CMAVE in 1997 to study microsporidia under the supervision of James Becnel and Chris Geden. He graduated with a Ph.D. in May 2002.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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